MICROSATELLITE MARKER BASED CHARACTERIZATION OF INDIGENOUS PIGS OF KERALA

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DECLARATION

I hereby declare that this thesis entitled, **"MICROSATELLITE MARKER BASED GENETIC CHARACTERIZATION OF INDIGENOUS PIGS OF KERALA"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled, **"MICROSATELLITE MARKER BASED GENETIC CHARACTERIZATION OF INDIGENOUS PIGS OF KERALA"** is a record of research work done independently by Dr. Ariprasath K. under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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CONTENTS

LIST OF TABLES

LIST OF PLATES

Introduction

1. INTRODUCTION

India harbors about 15% of world's livestock population, of which the pig population is 13 millions. Eighty four percent of the total pig population is indigenous. As per the Vedic literature (Smrti sastra), Vishnu's third incarnation is called 'Varaha', a boar and is believed that he descended into the waters to draw up the earth with the help of the tusks. In earlier days pigs were treated as holy creatures, the physical features of which were well described in Mahabharata.

Domestication of pigs took place in Europe (*Sus scorfa*) some where around the Baltic seas in the Neolithic times. In Asia, pigs (*Sus vittatus*) were separately domesticated in China around 3000 BC. These pigs were separated from the Middle and East Asian mainland extending from western India to central China and some of the islands like Japan, Sumatra and Borneo. In 1857 AD, the use of pig lard in preparation of cartridges by British, resulted in the famous 'Sepoy Mutiny', a pioneer attempt to Indian freedom fight.

The native pigs are small in size, pot bellied, hairy and black in colour with long face and short ears. These animals are unique in characters like disease resistance, lean meat production, and bristle yield. Phenotypic variation is noticed among the native pigs of different localities. These pigs have early age at maturity and shorter farrowing interval and poor average litter size: 5.2 at birth and 3.0 at weaning.

Pig rearing in India has been a traditional occupation of weaker sections of rural society. In II and III Five Year Plans, co-ordinated programme for piggery development was implemented in many states. Meat from all types of pigs is consumed in India, but people in north-eastern India prefer black pigs for meat purpose.

Natural and artificial selections over centuries have resulted in breed evolution and germplasm improvement. Various breeds are developed suitable for different environmental conditions. For instance, certain breeds are adapted to withstand extreme heat, others are especially resistant to disease, and many others are adapted to survive periods of draught – implying he rule of 'survival of the fittest'.

Species describes a group of animals or plants that have similar characters. Within each species, there are many different subgroups called breeds sharing certain unique traits. For e.g.: White Berkshire and Tamworth hogs both belong to the domestic pig species (*Sus scorfa*). Berkshires have thick black hair, while Tamworth pigs are known for their golden red colouring.

Various breeds of pigs exist in India, and are not yet properly studied and characterized. The population sizes of these breeds are decreasing in recent years, because of lower growth rate, feed conversion efficiency and lean meat percent than European and American breeds. Special efforts are therefore required to conserve the genetic resources of these local breeds. A systematic evaluation of genetic diversity of these important breeds will enable us to understand and formulate a rational plan for their conservation.

The native pigs are evolved by nature, they are resistant to various diseases and can withstand wide range of environmental extremities. There is potential to identify genes for disease tolerance and other adaptive traits such as heat tolerance in wild/indigenous life and these genes can be transferred to otherwise vulnerable stock by marker associated introgression.

As a result of industrialization and globalization, the indigenous animals are challenged with crossbred animals which are commercially superior. For preserving the biodiversity, those animals need to be conserved either in seed banks or *in situ* where ever possible and desirable. As a preliminary step, these germplasm have to be properly characterized and grouped based on various physical and molecular markers. Molecular tools offer better means to characterize a breed in terms of variation at loci associated with phenotypes. This data will be useful in preserving a representative gene pool of the populations.

For the purpose of conservation and exploitation of the superior nature of the native breeds it is necessary to take up genetic studies to find out how much related the individuals are within and between population. This can be done using several morphological, chromosomal and biochemical markers such as blood polymorphisms.

Presently the DNA markers are found very effective and offer maximum information about a population. After the advent of PCR (Polymerase Chain Reaction) technique, the DNA markers such as Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPD) and Variable Number of Tandem Repeats (VNTR) are found useful in genetic diversity studies.

In the past few years microsatellite markers have become the most popular molecular marker system and have been intensely applied in genome mapping, biodiversity and phylogeny studies of livestock. These markers are multi-allelic, more polymorphic and cheaper to genotype. Multi-allelic marker systems are more informative and powerful than a di-allelic marker system. Some of these markers are found associated the Quantitative Trait Loci (QTL) and thus aiding in Marker Assisted Selection (MAS) of the breeding stock.

Cattle and pigs have thousands of microsatellite markers as compared to hundreds in poultry and sheep. The molecular data of various species is organized in specific databases and can be accessed via the internet. The dense maps of cattle and pig cover the genome at approximately five centi Morgan level and provide the infrastructure needed for both for linkage and physical maps. The genetic linkage map of pig has over 2000 loci including approximately 300 genes.

Even though many studies have been conducted on desi pigs, characterization using DNA markers are not yet carried out. Hence the present study is envisaged with the following objectives.

1. To determine the allele frequency, heterozygosity and polymorphic information content of the selected microsatellite markers in desi pigs of Kerala.

2. To evaluate genetic differences in different population based on the polymorphism of microsatellite markers.

Review of Literature

2. REVIEW OF LITERATURE

2.1 GENETIC DIVERSITY

The food and agriculture organization (FAO) estimated that there are approximately 5000 different breeds of animals available in the world. Unfortunately one third of them are endangered and one becomes extinct per week. Contrary to the situation in plant genetic resources approaches for characterization and conservation of these domestic animals are strikingly less. The world is full of diverse variety of excellent animals, the potential of which is not accounted. Taihu pigs are from China, eat a high proportion of forage food and produce litter of up to 16 piglets compared to 10 for most western breeds. The number of these pigs has reduced to a great extent and is an endangered species now. Many breeds are being exported to other countries and are conserved and improved there. For example the highly prolific Chinese breeds and Iberian pigs were exported to developed countries, even the genes for their superior traits were targeted to be introduced into the industrial genetic lines (Pereira, 1998).

There is now increasing evidence that local breeds are superior to exotic ones in adaptability and their productiveness can be improved by crossbreeding within reasonable timeframe. In developing countries, the native pigs, for example Griollo type pigs in Mexico was almost replaced by imported white pigs despite of its usefulness for small scale holders & its ability to make use of local feed and its better taste (Anderson, *et al.* 1999).

Global programmes have to be implemented to determine the genetic distances among livestock breeds. Notter (1999) suggested establishing 'core collections' of diversity within each species to ensure the full range of genetic variation within the species. He also mentioned that different molecular techniques

can be employed to quantify the relationship among breeds in analyzing farm animal genetic diversity.

The genetic diversities and relationships of four Chinese indigenous pig breeds and one Australian commercial pig breed have been evaluated using 27 microsatellite markers recommended by the International Society of Animal Genetics (ISAG) and FAO. The results do not agree completely with the traditional classification of Chinese pig breeds (Li *et al.*, 2000).

The number of breeds of pigs exceeds 600 world wide. The wild boars and domestic pigs considerably differed from each other; the differences were studied widely in Europe & Asia (Giuffra *et al.* in 2000).

The study using 10 microsatellite loci conducted in 117 unrelated Mexican hairless pigs revealed that these pigs had developed several genetically distinct lines associated with their geographic location. It was also concluded that the Mexican Creole Pig population was a reservoir of genetic diversity and was important to preserve and evaluate the lines as a source of new alleles for the future improvement of commercial pig lines (Lemus *et al.*, 2001).

Eding *et al.* (2002) defined genetic diversity of a set of animal populations as the maximum genetic variance that can be obtained in a random mating population that was bred from that set of populations. They found a method for making quantitative assessment of contribution of different breeds to genetic diversity, which was very useful in planning for genetic conservation.

The Wuzhishan pig one of the special and rare small pig breeds in the world were characterized using serum protein polymorphism. The results showed that the Donglan pig and Baixiang pig was the closest where as the Lantang and Landrace was the farthest and that the Chinese breeds could be divided into three branches.

The genetic variation of Yimeng black pig and Duroc were the highest and the lowest, respectively. The genetic variation of Chinese indigenous breeds was much more than that of exotic groups. The results also supported the geographic distribution of Chinese indigenous pig breeds in certain extent (Mo *et al.*, 2003).

26 microsatellite markers were employed to analyze the genetic diversity of 18 Chinese indigenous pig breeds. The results implied that most of the genetic variation was within breeds and only a minority existed between breeds. The number of alleles in each locus across the population ranged from 8 to 31 which were generally higher than that of the European pig populations. The genetic distances between these breeds based on the microsatellite markers are low, indicating these Chinese indigenous pig breeds have close relationships and might have originated from a common ancestor (Yang *et al.*, 2003).

The microsatellite markers had been recommended by the Food and Agricultural Organization and the International Society for Animal Genetics (FAO-ISAG) to study the diversity of global pig breeds. 572 unrelated Chinese indigenous pigs and 184 unrelated exotic pigs of 10 breeds were selected, and the genetic analysis study was performed using 26 microsatellite markers. The cluster results were consistent with their geographical distribution and agreed with the "Book of pig breeds in China", an official document of Chinese pigs (Li *et al.*, 2004).

536 blood samples were collected from 10 pig breeds viz. Wuzhishan miniature, Diannan Xioioer, Guizhou miniature, Yimeng black, Hanzhong black and Rongehang, Erhualian Jinhua and Bama Xiang. The inter and intra population variation analysis using 10 microsatellite marker revealed the first 3 breeds formed one group, second three formed another group and the last 3 breeds form yet another group (Wang *et al.*, 2004).

2.2 MOLECULAR MARKERS

The markers revealing variation at DNA sequence level are called molecular markers or genetic markers. The satellite DNA is characterized by huge array of the short or long repeats spanning several millions of nucleotide and is the typical centromere sequences in many mammals (Singer, 1982).

The apparently non-functional DNA is either the single copy DNA or the repetitive DNA. The repetitive elements may be interspersed in the genome or may occur as tandem repeats (Schmid and Jelinek, 1982). In mammals, two major groups of the interspersed repetitive elements can be recognized: the short interspersed nuclear elements (SINEs) and the long interspersed nuclear elements (LINEs) (Schmid and Jelinek, 1982; Rogers, 1983; Singer and Skorownski, 1985). About 5-10% of structural loci are polymorphic in mammalian genome as determined by electrophoretic techniques (Smith and Simpson, 1986).

The repetitive elements arranged in tandem are as common as the interspersed repeats. The tandem repeats are broadly referred to as the satellite type of DNA. The satellite types are further classified according to the size or location of the repeats telomeric satellite, minisatellite and microsatellite DNA (Brutlag, 1980; Prosser *et al.*, 1986).

The hypervariability of the simple sequence stretches were due to slippage events occurring during DNA replication and these are flanked by unique DNA sequences enabling the amplification using PCR (Tautz, 1989).

Several molecular markers like AFLP, RAPD and microsatellites have been used extensively for the analysis of genetic diversity in different livestock species (Saitbekova *et al.*, 1999; Ajmone-Marsan *et al.*, 2001; Arora *et al.*, 2003; Suprabha, 2003; Bhattacharya *et al.*, 2004).

Various molecular markers including RFLPs, (Milkawa *et al.* 1999; Wang *et al.*, 2002), RAPDs (Liu *et al.*, 1996), VNTRs (Singer *et al.*, 1996) and microsatellite markers (Olliver *et al.*, 1989; Ruyter *et al.*, 1993; Hoyheim *et al.*, 1994; Jung *et al.*, 1994; Winter *et al.*, 1994; Olliver *et al.*, 1995; Fronicke *et al.*, 1996; Gong *et al.*, 1996; Kapke *et al.*, 1996; Li Ning *et al.*,1996; Rohrer *et al.*1996; Wu *et al.*, 1996; Milkawa *et al.*, 1999; Leeb *et al.*, 1999; Zhao *et al.*, 1999) reported in pigs. Microsatellite markers for characterization of different pig breeds were attempted as early as 1992 (Johansson *et al.*, 1992; Coppoeters *et al.*, 1993, Fredholm *et al.*, 1993; Sun *et al.*, 1996; Duscher *et al.*, 1998).

2.2.1 Microsatellites:

The dinucleotide repeat sequence (dA-dC)n (dG-dT)n represents one of the most abundant families of interspersed repetitive DNA in eukaryotic genomes (Miesfield *et al.*, 1981). Microsatellites sequences have been found in all eukaryotes examined but not in prokaryotes (Hamada *et al.*, 1982).

Variation of a dinucleotide repeat unit in a malarial parasite antigen (Kemp *et al.*, 1987, Weber, 1988) prompted the study of other (dA-dC)n (dG-dT)n sequences, and it was found that the repeats are polymorphic and informative (Weber and May 1989, Smeets *et al.*, 1989). The existence of short tandem repeats such as d(A)n, d(T)n,(dT-dC)n and (dG-dT)n have also been described by several investigators (Tautz, 1989, Litt and Luty, 1989). These loci can be used as highly polymorphic genetic markers, based on the variability in length of the short tandem repeat sequences (Weber and May, 1989; Litt and Luty, 1989).

The microsatellites show site-specific length variation and hence, on average, a PIC of 0.61, and make them versatile markers for genome mapping. PIC calculated from the allele frequencies in the population was found to be positively

correlated with the length of repeat (Weber 1990a) *i.e.* for longer repeats more alleles are expected.

According to Troyer *et al.* (1990) microsatellites were extremely repetitive short sequences with repeats of two or three bases long located at every 10,000 bases of DNA sequences and that they could be used to assess the degree of inbreeding in populations, to identify animals heterozygous for simple recessive traits and in gene mapping research.

Weber (1990) examined over 100 human (dC-dA)n, (dG-dT)n sequences and divided the sequences into three categories: perfect repeat sequences without interruptions in the runs of CA or GT dinucleotides (64%), imperfect repeat sequences with one or more interruptions in the run of repeats (25%), and compound repeat sequences with adjacent tandem simple repeats of a different sequence (11%).

The function of the (CA)n blocks is unknown, but there are reports which indicate that these sequences could enhance the expression of an adjacent gene especially when the repeat sequence is close to the promoter sequence (Glaser *et al.*, 1990). It is also proposed that these repeat sequences serve as hotspots for recombination. The absence of (CA)n repeats in prokaryotic genomes suggests that they might have a role in packaging and condensing DNA in eukaryotes. Moreover, the expansion of trinucleotide repeats as the cause of dominantly inherited neurodegenerative disorders (La Spada *et al.*, 1991) assigns a pathogenic role to some microsatellites.

A striking feature of the genomic organization in the eukaryotes is that the coding sequences constitute only a minor portion, about 5 to 10 percent in the mammals, of the total genome (Hochgeschwender and Brennan, 1991).

Stallings *et al.* (1991) determined the distribution of GT repetitive sequences in the human genome by analyzing over 3700 cosmid clones containing human DNA and found that on average, a GT repetitive sequences occurred every 30kb in DNA from euchromatic regions and were significantly underrepresented in certain heterochromatin.

Microsatellite sequences can be amplified *in vitro* using PCR or can be analysed in a similar fashion to minisatellite probes (Haberfeld *et al.*, 1991). By PCR, these polymorphisms were revealed by amplification of genomic DNA using primers flanking the chosen repeat sequence and specific for a given locus (Cornall *et al.*, 1991; Kemp and Teale, 1991). The alleles (which typically differ in length by 21 bp) can be differentiated by fractionating the amplified DNA product on a high resolution gel. Automated DNA typing methods have been developed which simplify detection and analysis of microsatellite polymorphism (Ziegle *et al.*, 1992).

On average, microsatellites occur in a haploid mammalian genome as 5-10 X 10^4 individual islets. If these islets were randomly distributed over the 3 X 10^9bp of the genome, one would expect a microsatellite sequence in every 50-100 kb on average (Weissenbach, 1993).

According to Moore *et al.* (1992) DNA microsatellites were important markers for gene maps in many species. The ease of isolation and characterization of these sequences coupled with the high levels of polymorphism and the method of analysis made them attractive gene markers.

The microsatellites are the repeat motifs of 1 to 6 base pairs repeated up to a maximum of about 100 times. They appear abundant and are evenly distributed throughout the genome occurring once in about every 6 kb of the genome. The most common microsatellite in the mammals are the (A)n, (CA)n, (AAAT)n and (AG)n (Beckmann and Weber, 1992).

Steffen *et al.* (1993) screened a partial plasmid library with bovine genomic inserts of about 500 base pairs with a (dC-dA)n, (dG-dT)n oligonucleotide probe for the repeated nucleotide motif (CA)n. Eleven positive clones were discovered and were subsequently isolated and sequenced.

The mutation rate in microsatellite is reported to be as high as 10^{-3} to 10^{-4} per locus per generation (Weber and Wong, 1993), where as the normal rate is 10^{-9} per nucleotide per generation (Crow, 1993).

Even though no specific function has been assigned to microsatellite sequences, they have proved to be efficient markers for the mapping of economic trait loci (Georges *et al.*, 1993) and disease genes in animals (Holmes, 1994). Thus these markers have proved to be of great use for studies in population and genetic characterization, ecological genetics, gene mapping and medical genetics and are the favoured markers in human and animal genetic research. The dinucleotide repeats may be located within the protein coding regions, within introns or between genes and are inherited in normal Mendelian fashion.

It was also found that microsatellite repeats with less than 10 repeat units are likely to be the monomorphic. On the other hand, the repeats with an average number of repeated units exceeding 20 may possess the polymorphism information content values of 0.6 or more (Vaiman *et al.*, 1995).

Microsatellite markers being highly polymorphic are widely used as efficient tool for parentage verification in several species. Usha *et al.* (1995) proved their supremacy over other markers in different species for parentage verification.

The degree of polymorphism and heterozygosity detected by microsatellites is much greater than that of protein markers (Arranz *et al.*, 1996).

The amount of polymorphism is directly proportional to the mutation rate. Since the microsatellites are new length variants the mutation rate is appreciable at these loci and the idea was confirmed by direct observations of the spontaneous events of the germline mutation from the pedigree analysis (Goldstein and Pollock, 1997).

Microsatellite markers are described in pigs all over the genome. Two microsatellite markers namely KS3, KS4 are reported on chromosome 6 (Rohrer *et al.* 1997) located at 110.4 cM and 59.8 cM.

Location and inheritance of four highly polymorphic porcine microsatellite markers KS5, KS6, KS7 & KS8 were confirmed in 7 full sib families with a minimum of 5 alleles (Rohrer, 1998).

Various DNA markers consisting of 237 microsatellites, 5 PRE-1 and one RFLP were reported all over the porcine genome to the total length of (sex averaged map) 2561.9 cM and the average distance between two markers was 11.4 cM. These include the clusters affecting growth and fatness on chromosome 4. The linkage map of all the chromosomes except for chromosome 1 was found to be longer in females than males. The average ratio of female to male recombination was calculated to be 1.55 (Mikawa *et al.*, 1999).

The microsatellite polymorphism have been well documented in fish, poultry and the livestock species including the pigs (Crooijmans *et al.*, 1997; Yang *et al.*, 1999; Diez-Tascon *et al.*, 2000; Bjornstad and Roed, 2001; Canon *et al.*, 2001).

In aiming to identify genes/genetic regions responsible for quantitative traits, a swine reference population was constructed using 31 microsatellite markers from porcine chromosome (SSC) 2, 4, 6 & 7 in Chinese local breeds. It was found that the female maps were longer than the male maps on SSC 2, 4 & 7 but female map of

SSC 6 was shorter than the male map. On SSC 7, a comprehensive linkage map was slightly longer than the skeletal map. SW2155, SW859 and SW1856 were rearranged to SW2155, SW589, SW859 (Su *et al.*, 2002).

Heterologous microsatellite markers *i.e.* markers derived from one species and tested in another species can reveal systemic relationship among different species that have been conserved during evolution (Arora *et al.*, 2003).

The effect of heterosis in Yorkshire and Meishan pigs were studied using 39 different microsatellite markers linked to growth traits. Markers were selected in about 20-30 cM distance from all over the genome and significant relationship was observed between genetic variability, extent of heterosis and heterozygosity for microsatellite markers (Zhang *et al.*, 2004).

2.2.2 Microsatellite markers associated with Quantitative Trait Loci (QTL):

In 1997 Wang *et al.* discovered 4 highly polymorphic porcine microsatellite loci in American pigs. QTL on chromosome 13 have been reported responsible for birth weight and early growth in piglets (Yu *et al.*, 1997). Kacirek *et al.* (1997) from Ohio state university analyzed the microsatellite variation in Yorkshire, Large White and Hampshire.

Different QTL affecting ovulation rate, located in human chromosome (HSA) 4 have been physically mapped chromosome 8 in pigs. Different human genes like fibroblast growth factor basic (PGF2; HSA4q) Phospho diestrase 6B (PDE6B; HSA4 p16.3) and amino peptidase S (PEPS; HSA4p 11-q 12) were investigated in porcine chromosome also and were physically mapped to porcine chromosome 8q 2.2-2.4, 8p1.1-1.2, 8 p 2.3 and 8p1.1 respectively (Rohrer, 1999).

Different markers for *E.coli* resistance in pigs have been reported by Meijerink *et al.* (2000). Physical mapping of different critical QTL in pigs was done

by Tornsten (2000) in Sweden. The genome scan assigned the gene responsible for belt formation to the centromeric region of chromosome 8. Using florescent *in situ* hybridization (FISH) various QTL were mapped, the marker SWC9 is found very useful in parentage verification in pigs.

A QTL on pig chromosome 4 affecting fatty acid metabolism was discovered in the Iberian and Landrace population (Perez-Enciso, 2000). The Iberian genotypes were fatter, slower growing and had lower linoleic acid and higher oleic acid percentage than Landrace genotype. The highly significant QTL for linoleic acid percent was mapped to 86 cM on chromosome 4 which is nested to fatness QTL. QTL for back fat thickness and back fat weight and Longissmus Dorsi area were mapped to the same position as the linoleic acid content QTL.

Scanning of porcine genome using microsatellites was carried out to detect QTL for back fat thickness (BFT) in the pig population. The economically important three QTL markers were located at various regions of the porcine genome, one between marker loci S0113 & SW 130 in chromosome 1 and other two QTLs on chromosome 13 & 18 respectively (Wu *et al.*, 2002).

The porcine chromosome 7 was investigated for various QTLs responsible for different economically important traits like growth and carcass traits. A QTL with highly significant effect on body weight was mapped at position 3 cM near the marker SW1343. Another QTL with impact on carcass length and dressing percentage were mapped at position 80 cM and 138 cM respectively. At 88 cM there were indications of QTL affecting moisture in muscle Longissimus dorsi (Zuo *et al.*, 2004).

Attempts have been made for detection and characterization of QTL for meat quality in pigs (Kim, 2005). These markers provide valuable information for subsequent QTL analysis and marker assisted selection. The microsatellite markers

for characterization based on the QTL for fatness in black pied and Large White pigs have been reported by Alfonso and Arana, 2005.

Microsatellite markers associated with QTL in pigs such as growth, carcass and meat quality traits were studied in two generation family of commercial lines. The 14 markers genotyped for the whole pedigree had an average heterozygosity of 0.61 in the parental generation and jointly are very efficient for parentage testing with an exclusion probability of 0.999 (Chen *et al.*, 2006).

2.2.3 Genetic Analysis of Closely Related Breeds/Populations:

Darwin (1868) observed that the pigs found in Bengal measured 44" height, where as the European breeds never exceed the height of 36". He also noted *S.indicus,* popularly known as Chinese pigs differ from *S.scorfa* in the skull morphology. He concluded that the native breeds of British province were rapidly disappearing and were replaced by improved breeds crossed with *S.indicus.*

Recent developments in molecular biology and statistics have opened the possibility of identifying major genes for genetic improvement of livestock with different molecular markers and statistical analysis procedures as described by Montaldo *et al.* (1998).

Sliva (1999) attempted to form a nucleus of local pigs and to characterize them morphologically by comparing the existing genotypes with the Bisaro pig population described 150 years ago. The results confirmed the utility of studying visible effects and polymorphisms for the genetic evaluation and characterization of population.

The allelic frequency data obtained after the PCR based genome scoring were utilized for studying the evolutionary relationships of the closely related breeds/ populations of a species or closely related species. A set of 11 European pig breeds

including a small sample of wild boars were chosen for genetic diversity studies, using 18 different microsatellite markers (Laval *et al.*, 2000) and confirmed that the genotypic frequencies generally agreed with Hardy-Weinberg expectation with average breed heterozygosity varying from 0.35 to 0.60.

The miniature pig breed, the Tibetan pigs distributed in remote mountain areas of China, have desirable meat qualities, small body size and easy manageability. The comparison of mean heterozygosity indicated that the within breed variation of Tibetan pigs is largest between breeds (Li *et al.*, 2000).

Researchers tried to measure the evolutionary change by counting the number of nucleotides that differ between same genes in animals of two different breeds (Giuffra *et al.*, 2000, Kijas and Andersson, 2001). They implemented the same technique in pigs and found effective for characterization. The phenotypic diversity and the microsatellite markers linked to these characters in farm animals were analyzed elaborately by Anderson (2001). Kaul *et al.* (2001) from NBAGR evaluated the genetic variability of 13 microsatellite markers in northern and north eastern Indian native pigs. They used the markers S0005, S0068, S0090 & S0218 markers and proposed these markers can be used with reliability for studying the genetic diversity and for identification of individuals in Indian pigs.

Analysis of the genetic variation of an endangered population is an important component for the success of conservation. On assessment of Romanian pig breeds significant heterozygosity was observed in 5 of the 10 loci. It was suggested the genetic markers such as CAST, PRLR or MC4R are useful for accelerating genetic progress in traditional breeds and established commercial lines (Ciobanu *et al.*, 2001).

The allelic diversity of KIT locus encoding the mast and stem cell growth factor receptor, as the prime cause for coat color in pigs has been studied extensively

by Pielberg *et al.* (2001). It was concluded that the presence of multiple KIT alleles in white pig breeds was due to a high mutation rate. They revealed extensive genetic diversity at dominant white locus in Landrace & Large White Yorkshire breeds. The dominant white may be due to strong selection for white color for more than 100 years.

Ten microsatellite markers have been recommended specially for parentage verification in pigs (Nechtelberger *et al.,* 2001). With the use of dye end labeled primer, it was concluded that it was important to select markers with a high degree of polymorphism in order to achieve a high probability for exclusion of false parentages.

About 58 different pig breeds were analyzed using 148 AFLP bands and 50 microsatellite loci by Sancristobal *et al.* (2002). Analysis of within and between breed variability was performed and two genetic distances were compared, namely DR (Reynolds) and DS (Standard Nei). It was found that the local breeds showed more dispersion. On comparison of the two genetic distances it was concluded that the contribution to European breeds from local, international and commercial lines were 49.6 %, 9.0 % and 32.0% respectively.

Type I DNA markers were used for genetic characterization of two Portuguese swine breeds (Ramos, 2003). Each breed consisted of two allelic variants of MC1R (melano cortin 1 receptor) allele EP^1 observed in white, spotted and belted pigs represented by Pietrain, Hampshire & Berkshire. The EP^2 allele was associated with dominant black color of Hampshire pigs.

Genetic variation and relationships of 18 Chinese indigenous pig breeds were tested by Shu-lin *et al.* (2003) using 27 different microsatellite markers. 92.14% of the individuals were clustered with their own breeds, and confirmed that these markers were very useful for breed characterization. It was concluded that the 18

Chinese indigenous breeds may have one common ancestor. The heterozygosity of 18 microsatellite markers were studied in as large as 1004 indigenous pigs of five breeds and 184 commercial pigs of three breeds in China. The observed heterozygosity, unbiased expected heterozygosity and the observed and effective number of alleles were used to estimate the genetic variation of each indigenous breed. Few small sub clusters were also found, which included 2-4 breeds each.

By observing wide variation in fecundity of pigs, QTL markers were traced and detected on chromosome 8, which influenced various reproductive traits in pigs (King, 2003). The study was conducted in one of the most prolific breeds, the Meishan pigs, using various markers associated with AREG & SLIT 2 gene. The QTLs were mapped in q arm of chromosome 8, one at 49 cM (near SLIT) and the other one at 100 cM (near AREG).

Single Nucleotide Polymorphism (SNP) is the most recent and accurate molecular tool for characterization and was adopted in different pig populations for QTL analysis (Jungerius, 2004). About 1545 SNPs are available for various purposes including characterization.

142 porcine microsatellite markers covering all chromosomes with average spacing of 15 cM were selected and studied for characterization, linkage & parentage verification by Schwarz (2005).

The loci linked with body growth were studied in Chinese indigenous Meishan pigs. Significant effects of heterozygosity in three growth traits were detected (Jiang *et al.,* 2005).

A group of feral pigs were selected from a wide area of Australia and Papua New Guinea, and were analysed using different microsatellite markers. The results revealed that 41% of the alleles were not represented in the genotypes from the

commercial pigs and that the commercial pigs had only seven alleles and not represented in the wild populations (Spencer, 2006).

2.2.4 Swine Markers:

As compared to the most other types of the DNA sequences, the microsatellites are highly polymorphic and are excellent genetic markers (Goldstein and Shiotterer, 1999). Besides, the obvious advantage of PCR based analysis, the applicability of the microsatellite markers in genome analysis primarily depend on the three inherent properties: abundance, hypervariablility and Mendelian inheritance. These properties make the microsatellites very informative markers in the genome analysis and are used for various applications in pigs and other species (Beuzen *et al.*, 2000; Ellegren, 2000b).

2.2.4.1 S0005 **:**

This microsatellite marker is located in chromosome 5 and the maximum number of alleles recorded is 11 with the size ranging from 203-243 bp (Fredholm *et al.*, 1993). The proposed heterozygosity is 100 with relative position at 88.2cM. The primer works well at 58°C although the proposed annealing temperature is 60°C (Rohrer *et al.*, 1996).

2.2.4.2 S0101 **:**

This marker is mapped to the short arm of chromosome number 7. About 13 alleles have been found varying in size from 196-224 bp (Rohrer *et al.*, 1996). The heterozygosity recorded is 100 and annealing temperature of 58°C by Alexander *et al.* (1996).

2.2.4.3 SW1026 :

This dinucleotide repeat microsatellite marker is mapped in 2nd chromosome at about 60.6 cM distance (Rohrer *et al.*, 1994). Rohrer *et al.* (1996) observed 13 alleles of size differing from 97-119 bp with 80% heterozygosity with the annealing temperature of 60°C.

2.2.4.4 SW2517 :

Alexander *et al.* (1996) mapped this dinucleotide repeat in 16th chromosome. As many as 22 alleles have been registered varying widely in size from 146-197 bp. The heterozygosity is around 90 and annealing temperature is 58°C (Rohrer *et al.*, 1996). This marker was recommended for parent verification (Schwarz *et al.*, 2005)

2.2.4.5 S0008 :

Fredholm *et al.* (1993) mapped this microsatellite to chromosome 1 with 90% heterozygosity and recorded number of alleles as 15 with a size range of 172-191 bp. Annealing temperature is ranged between 58-62 °C (Rohrer *et al.*, 1996). This marker was also used for characterization of pigs (Mikawa *et al.*, 1999).

2.3 ISOLATION OF GENOMIC DNA

The isolation of pure, high molecular weight genomic DNA is the initial step to carryout any genetic analysis using DNA marker polymorphisms. Although several methods have been described for isolation of genomic DNA from different sources like blood, tissue, semen etc the phenol-chloroform extraction method is the widely adopted one.

Blin and Stafford (1976) described a method of DNA isolation adopting phenol extraction from various tissues like calf thymus and human placenta.

Standard protocol involving Proteinase-K digestion and phenol:chloroform extraction followed by ethanol precipitation is an efficient method for isolation of genomic DNA from whole blood (Andersson *et al.*, 1986; Oliver *et al.*, 1989; Sambrook *et al.*, 1989; Trommelen *et al.*, 1993; Arora *et al.*, 2003; Chenyambuga *et al.*, 2004).

Aravindakshan *et al.* (1998) compared three methods of DNA extraction namely, the guanidine hydrochloride method, the high salt method and the phenol-chloroform method and demonstrated that both high salt method and phenol-chloroform method yield good amount of high molecular weight DNA from cattle white blood cells when the guanidine hydrochloride method failed to yield pure DNA.

Use of Proteinase K for digestion, followed by salting out for obtaining genomic DNA from blood and semen was described by Arranz *et al.* (2001).

2.3.1 Yield and Purity of DNA:

Apparao *et al.* (1994) extracted genomic DNA from cattle, buffalo, sheep, goat and swine using a modified phenol-chloroform extraction method and obtained yields of 250 to 300 µg DNA from 15 ml of whole blood.

Senthil *et al.* (1996) obtained 615.55 ± 0.72 µg and 444.58 ± 21.54 µg DNA using high salt method and phenol chloroform method, respectively, from 15 ml blood with optical density (OD) ratio (260/280 nm) of more than 1.7.

Aravindakshan *et al.* (1998) reported that the mean yield of DNA extracted from 10 ml of whole blood of cattle and buffalo by phenol and high salt methods were 394.50/446.16 µg and 344.25/432.83 µg respectively. The ratio of optical densities (OD) at 260 and 280 nm was consistent between 1.75 and 1.91 indicating good deproteinisation.

2.4 POLYMERASE CHAIN REACTION

The polymerase chain reaction, involves the *in vitro* enzymatic synthesis of millions of copies of specific DNA segments, has transformed the way DNA analysis is carried out in molecular studies. Microsatellite markers have become the markers of choice for population genetic studies mainly because of the possibility of combining their analysis with the PCR.

Mullis *et al.* (1986) demonstrated the exponential increase in the concentration of a specific DNA sequence involving the reciprocal interaction of two oligonucleotides and the DNA polymerase extension products, the synthesis of which they prime, in repetitive cycles of denaturation, hybridization and polymerase extension.

Eckert and Kunkel (1990) demonstrated that despite lacking a 3′-5′ proof reading exonuclease, the *Thermus aquatics* (Taq) DNA polymerase could catalyse highly accurate DNA synthesis *in vitro.* It was shown that the error rate per nucleotide polymerized at 70°C was as low as 10^{-5} for base substitution and 10^{-6} for frame shift errors. It was also reported that the frequency of mutation responded to changes in dNTP concentration, pH and $MgCl_2$ concentration.

A computer program was designed by Lowe *et al.* (1990), which could rapidly scan nucleic acid sequences to select all possible pairs of oligonucleotides suitable for use as primers for direct and efficient amplification of DNA by PCR. Several such programmes are available online for researcher's reference.

Erlich *et al.* (1991) reviewed the spectrum of advances made possible by the PCR technology and enumerated several applications including construction of

genetic maps, reconstruction of evolutionary history of species, foot printing, gene expression studies and diagnosis of diseases.

Don *et al.* (1991) described a 'touchdown' PCR strategy to bypass spurious amplifications frequently encountered in PCR. Saiki *et al.*(1998) detailed that PCR involves merely combining DNA sample with oligonucleotide primers, deoxyribonucleoside triphosphates and the thermostable *Taq* DNA polymerase in a suitable buffer, followed by repeatedly heating and cooling the mixture for several hours until the desired amount of amplification was achieved.

Optimization of PCR, especially the annealing temperature was essential for each primer pair. The annealing temperature was usually calculated as the temperature of melting (Tm) of the primers ±4°C but must be determined empirically. Another important condition to be optimized is the concentration of $MgCl_2$ in the reaction mix (Oh and Mao, 1999). Various types of PCR have been described for optimum amplification.

In nested PCR, two pairs of primers were employed for a single locus. It was found that the nested PCR was very reliable way to get better products, since the possibility of wrong locus to get amplified for the second time is very low. In this type, the product of first PCR can be used as template for the second PCR reaction with a new set of primers that are internal to the original (Verdin *et al.*, 2000).

When heterologous primers are employed, a set of PCRs can be run with different annealing temperatures in the same block. This saves time and used to fix the exact annealing temperature for primers (Padmakumar *et al.*, 2005). Gradient PCR was used for fixing the exact annealing temperature for primers SW 349, S008, S2517, S2509 and SW1026.

Multiplex PCR is a variant of PCR which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. Since its first description by Chamberlain et al (1990), this method has been applied in many areas of DNA testing, including analyses of deletions, mutations, and polymorphisms, or quantitative assays and reverse transcription PCR. Typically, it is used for genotyping applications where simultaneous analysis of multiple markers is required, as in detection of pathogens or genetically modified organisms (GMOs), or for microsatellite analysis. Multiplex assays can be tedious and time-consuming to establish, requiring lengthy optimization procedures (Duvigneau *et al.* 2005).

2.5 LABELING OF PRIMERS

Tautz (1989) end-labelled the PCR primers at the 5' end using γ^{32} P-ATP (1 μCi for 10pM primer) using T4 Polynucleotide Kinase (PNK). The amplified products were loaded on 6 percent denaturing polyacrylamide gels along side a sequencing ladder for determination of size differences between alleles.

Hughes (1993) described a method of typing microsatellite polymorphisms involving amplification by PCR. One primer of each pair was end-labelled with 10 μCi of γ^{32} P-ATP using T4 PNK, the end-labelling reaction was carried out at 37°C for 30 minutes. The alleles were separated by denaturing gel electophoresis and detected by autoradiography.

Hughes *et al.* (1993) described a technique of end labeling of single primer in 2.5μl reaction volume with 6pM primer and 10μCi of γ^{32}PATP using T4 polynucleotide kinase incubating at 37°C for 30 min. It is suggested that the use of a single end labeled primer was preferable for incorporating either ^{32}P or ^{35}S labeled nucleotide as it resulted in a simpler allele pattern because only the CA or GT strand is labeled and these two had slightly different mobilities.

Buchanan *et al.* (1994) labeled primers using [γ-^{32}P] ATP or [γ-^{33}P] ATP and T4 Polynucleotide kinase or employed direct incorporation of [α-^{32}P] dATP in PCR product and using both unlabelled primers for microsatellite analysis.

Kemp *et al.* (1995) used γ^{33}P or γ^{32}P for labeling one of the primers of each pair to amplify genomic DNA in 5 μl volumes of PCR amplifications. They also ranked the markers by judging the autoradiographs obtained on the basis of the ease of interpretation.

Arranz *et al.* (1996) used α^{35}S dATP in the amplification reaction and the genetic variants were visualized by autoradiography using the sequence of phage M13mp18 as a size standard.

End labeling is a rapid and sensitive method for radio active labeling of DNA fragments. Two commonly used methods for end labeling are the "fill-in" method using the Klenow fragment of *E. coli* DNA polymerase and the kinase reaction using the T4 polynucleotide kinase to transfer labeled phosphate to the 5′ end of the DNA molecule (Harwood, 1996).

Yang *et al.* (1999) used silver staining of gels for visualization and analysis of amplified DNA fragments.

Several other workers also accomplished labeling of PCR products with radioisotopes either by end labeling one of the primers with γ^{32}P ATP with PNK enzyme and by using the labeled primer in the PCR assay or by the direct incorporation of radio labeled nucleotides into the reaction mix (Russel *et al.*, 2000; Arranz *et al.*, 2001).

The primers labeled with the fluorescent dye like rhodamine can be used to confirm the amplified products. Following the amplification, the fluorescence correlation spectroscopy (FCS) developed in early 1970s, can be employed. This

method is easy to adapt and time saving compared with the radioisotope end labeled primer (Walter *et al.*, 1996).

2.6 DETECTION OF MICROSATELLITE POLYMORPHISMS

Microsatellites are difficult to type being short tandem repeats, alleles often differ in size by two or three base pairs, and additional shadow bands arise due to slippage during PCR amplification and the different mobilities of CA-rich and GT-rich strands (Hughes, 1993). The PCR products can be resolved on polyacrylamide sequencing gels (Yang *et al.*, 1999; Mukesh *et al.*, 2004; Kumar *et al.*, 2005) and sized using appropriate dideoxy sequencing ladders. The PCR products separated can be visualized by silver staining, autoradiography or by means of an automated sequencer.

Analytical polyacrylamide gels containing radioactive DNA may be fixed or dried before autoradiography. Drying of gels can be accomplished by using a commercial gel dryer after blotting the gel onto a piece of Whatman 3MM paper. Drying of gels is necessary when gels contain DNA labeled with weak β emitting isotopes like ^{35}S or small amounts of ^{32}P which require long exposures to obtain an adequate radiographic image (Sambrook *et al.*, 1989).

2.7 SEQUENCING

DNA can be sequenced by a chemical procedure that breaks a terminally labeled DNA molecule partially at each repetition of a base. The lengths of the labeled fragments then identify the positions of that base. Maxam and Gilbert, (1977) described reactions that cleave DNA preferentially at guanines, adenines, cytosines and thymines equally, and at cytosines alone. When the products of these four reactions are resolved by size, by electrophoresis on a polyacrylamide gel, the

DNA sequence can be read from the pattern of radioactive bands. The technique will permit sequencing of at least 100 bases from the point of labeling.

Sanger *et al.* (1977) described the chain termination method of DNA sequencing, where dideoxynucleotides were used for specific termination of the growing chain. One of the nucleotides was radiolabeled so that the mixture fractionated by electrophoresis could be subjected to autoradiography from which the required sequence could be read.

The use of $\alpha^{35}S$ dATP in DNA sequencing reaction produced very sharp definition of bands on a sequencing gel autoradiograph due to the sharp path length of β particles emitted by the isotope (Biggin *et al.*, 1983).

2.8 STATISTICAL ANALYSES

2.8.1 Estimation of Phylogenetic Relationship between Breeds

Because of the tremendous economic and cultural importance, much work was focused on the classification of pigs by assigning them to breed groups. The term "breed" is not uniformly applied in the classification of pig populations, but describes the pig population of a certain area with varying degrees of uniformity or express uniformity in a few traits affected by simple gene action such as colour, colour pattern, hair pattern, body morphology etc. The breeds in turn are subdivided into populations, strains or lines. Different breeds have existed as socioeconomic entities for some hundreds of years.

Understanding the extent and pattern of genetic variability among breeds may help in the development of more rational breeding programmes (Kidd *et al.* 1974) and is prerequisite to develop conservation strategies for genetic resources.

The best estimate of measuring genetic distance is obtained using genetically inherited polymorphism: blood groups, serum proteins and DNA markers. During the last few decades, blood group and protein polymorphisms have been used extensively for studying genetic distances in several animals including pigs (Naik 1978), but these markers were not always useful for classifying closely related populations because the extent of variation was often quite low.

The analysis of DNA sequences reveal the ultimate source of genetic variability, and thus offer highly informative markers for measuring genetic distances (Teale *et al.* 1994). Over the past decade, molecular genetic data have been used to address phylogenetic relationships. The D-loop region of mitochondrial DNA (mtDNA) has been found to be especially useful, because it is relatively short and highly variable (Avise *et al.* 1987). Genetic variation in hyper variable loci has been used extensively for inter population studies (Edwards *et al.* 1992). Microsatellite sequences have offered great potential for genetic comparisons within and between populations (Bowcock *et al.* 1994, Buchanan *et al.* 1994) and have facilitated the construction of phylogenetic trees of animals that reflect their geographical origin or relatedness (Loftus *et al.* 1994, Mengers *et al.* 1994).

Microsatellite loci have high mutation rates in comparison with other genetic markers (Weber and Wong 1993) and the average heterozygosity ranging between 30 and 80 percent. An understanding of the mechanism of mutations in microsatellite sequences is important if these markers are to be used in evolutionary genetics.

2.8.2 Genetic Distance

Genetic distance calculated using allele frequencies is a powerful method for detecting the effects of natural selection among different genetic groups. Measures of genetic distance provide the best available objective description of the genetic

differentiation between any two breeds. Estimation of genetic distance helps to identify genetically unique breeds so that they may be prioritized for breed conservation purposes. It also provides insights into the history of animal domestication.

Genetic distances are designed to express the variation between two populations as a single number (Smith 1977). The value gives a relative estimate of how distant the breeds are when compared to each other. If two populations have the same allele frequency distribution at the same locus the genetic distance between them at least for that locus, is zero. If the populations have no alleles in common at any locus the distance may be separated by the maximum value which can be infinite (Swofford and Olsen 1990). Genetic distances may be regarded as a means of comparing pairs of extant populations or as a basis for constructing evolutionary histories for the population.

According to Nei (1978), the number of individuals to be used for estimating genetic distance can be very small if genetic distance is large and average heterozygosity of the two species compared is low.

Genetic distances are used either for estimating evolutionary times or for constructing phylogenies (dendrograms). A distance measure that is appropriate for estimating evolutionary time is not good for obtaining the correct topology (Nei and Takezaki, 1994). Even though all the methods of calculation of genetic distances are based on allele frequencies, the assumptions made are different for each method.

Barker (1994) suggested that when pair wise genetic distances methods were used to construct a single phylogeny, all the relationships among the breeds are best represented. Such a phylogeny would aid in objective and rational decision making in the choice of breeds for preservation, utilization and evaluation studies to determine comparative genetic merit for production traits.

Takezaki and Nei (1996) compared various measures of genetic distance used for the reconstruction of phylogenetic trees from microsatellite frequency data and showed that the accuracy of Cavalli-Sforza and Edwards chord distance, D_C and Nei's D_A distance were generally higher than the other distances.

Boyce *et al.* (1997) used both allele size based method and frequency based method of genetic distance estimation in bighorn sheep and observed that even with good repeat loci, size based methods may be unduly influenced by one or only a few loci. It is also reported that the genetic distance values tended to increase withincreasing geographic distance.

Genetic distance is a measure of overall evolutionary divergence and serves as a useful tool for authentication of pedigree, for characterisation of different breeds or strains within a species and for evaluation of the change in variation in species over time (Mitra *et al.*, 1999).

Kalinowski (2002) proved from computer simulation studies that an equivalent precision of estimates of genetic distances could be achieved either by examining a few loci with many alleles or many loci with few alleles.

Vijh *et al.* (2004) estimated several genetic distance measures from microsatellite data in indigenous poultry germplasm using infinite allele model and used them for phylogenetic tree construction. They found that the tree obtained using Nei's D_A was the most appropriate as it could be backed by statistical criterion and a historical and demographic perspective.

Kalinowski (2005) analysed the rate at which increasing sample sizes decrease the coefficient of variation of genetic distance estimates. It was reported that the rate depends upon the value of F_{ST}, which is a measure of differentiation between the populations. It was noticed that when the F_{ST} was greater than 0.05,

samples of less than 20 per population was sufficient but when F_{ST} was less than 0.01 samples of 100 or more individuals per population would be useful.

2.8.3 Dendrograms or Phylogenetic Trees

Phylogenetic trees are graphical representation consisting of nodes (taxonomic units) and branches (pathway connecting nodes) that summarize the evolutionary relationships among breeds. There are many statistical methods for constructing phylogenetic trees or dendrograms from molecular data. They can be classified into distance methods and discrete character methods. Distance matrix methods are based on the set of distances calculated between each pair of taxonomic units. The configuration of the resulting tree depends on the quality of the distance measure.

Popular distance methods include unweighted pair group method with arithmetic means (UPGMA) Sneath and Sokal (1973) and neighbor-joining (NJ) approach was later developed by Saitou and Imanishi (1989) and Rzhetsky and Nei (1992). UPGMA depends on the assumption that the rate of evolution is the same for all evolutionary lineages, so that any true heterogeneity would remain undetected. But the NJ method allows unequal rates of evolution, thus eliminating such risks.

In the discrete character method, a tree is constructed by considering the evolutionary relationships of DNA sequences at each nucleotide site. Two major methods belonging to this category are the maximum parsimony (MP) method (Eck and Dayhoff 1966) and the maximum likelihood (ML) method (Felsenstein 1981).

The phylogenetic trees may be represented either as rooted or unrooted trees. A rooted tree conveys the options of ordering of the species on a tree, while an unrooted tree merely reflects distances between units with no notion of ancestral relationship.

The accuracy of the tree is dependent on the number of loci typed, nature of the samples used, the tree making method and the distance measure used for analysis. The species under study also has an effect. The populations that diverged for millions of years are to be regarded as different from those that diverged recently.

The species which are widely separated requires few markers for accurate comparison in contrast to the closely related species. Nei *et al.* (1983) have shown that even with 30 loci the constructed tree could be incorrect.

Saitou and Nei (1987) proposed the neighbour joining method for reconstructing phylogenetic trees from evolutionary distance data. The method finds pairs of operational taxonomic units (OTUs) or neighbours that minimize the total branch length at each stage of clustering of OTUs starting with a star like tree.

Later Nei and Takezaki (1994) worked out the confidence level of constructing trees using different number of loci by simulation. The percentage of replications for the correct tree topology varied between 82-96% with the different methods of genetic distance. If the true genetic distances are less than 0.004 and if there are many branches, the tree would be incorrect even with 100 markers. However the calculation of genetic distance is dependent on the number and frequency of alleles of the markers used for calculating the genetic distance.

The pair wise genetic distance values between breeds can be used to construct phylogenetic trees that makes possible to readily identify genetically unique breeds (Ruane, 1999).

The current intensive characterization of microsatellite polymorphism in a wide range of species suggests that these markers are very useful in population and ecological genetic studies.

Materials & Methods

3. MATERIALS AND METHODS

3.1 SOURCE OF SAMPLES:

The blood samples were collected from animals from different districts of Kerala, and from the animals maintained in university pig breeding farm, Mannuthy. Samples were collected from local slaughter houses also.. The physical features of the animals were noted. Animals below one year were preferred to overcome the bottlenecks in restraining and blood collection. The Meat Products of India (MPI), local markets in Koothattukulam and Ollur were also opted for blood sample collection. Samples collected from university pig breeding farm, Mannuthy were grouped as population I, those collected from Koothattukulam belonged to population II, samples from Ollur slaughter house were population III and the ones from other areas constituted group IV.

Morphologically the animals included in the study were of two types – one which is hairy, short faced and dished back (Plate I) and the other with less hair, long faced, pot bellied and non-dished back (Plate II).

3.2 BLOOD COLLECTION:

The physical features of the animal and place from where the samples are collected are recorded. About five ml of blood was collected using fresh syringe and needle from ear vein of animals maintained in farms and households. At the slaughter houses, the blood was collected from the jugular vein after stunning. The blood was collected with fresh EDTA (1mg/ml of blood) in vials and stored in igloo box at 4°C until reaching the laboratory. In the laboratory, the samples were stored in refrigerator until processing.

3.3 ISOLATION OF GENOMIC DNA FROM WHOLE BLOOD:

DNA was extracted from whole blood using the standard phenol- chloroform extraction procedure (Sambrook et al., 1989) with modifications. Five ml of blood collected in a 15 ml centrifuge tube was centrifuged at 4000 rpm for 10 min and the plasma was discarded leaving erythrocytes and leucocytes. Two to three volumes of ice-cold RBC lysis buffer (150 m*M* NH_4Cl, 10 m*M* KCl, 0.1 m*M* EDTA) was added, mixed well and kept in ice with occasional mixing for 10 minutes for complete lysis of erythrocytes. The leucocytes were recovered by centrifuging at 3500 rpm for 15 min. and the supernatant containing lysed erythrocytes was discarded. The above two were repeated till the cell pellet was clear without any unlysed erythrocytes. The cell pellet was washed twice with 10 ml of Tris buffered saline (TBS-140 m*M* NaCl, 0.5 m*M* KCl, 0.25 m*M* Tris) by vigorous vortexing followed by centrifugation at 3000 rpm for 10 minutes. The washed white cell pellet was resuspended completely by vortexing in 5 ml of saline EDTA buffer (SE-75 m*M* NaCl, 35 m*M* EDTA). To this mixture 25 μl of proteinase-K and 0.25 ml of 20 per cent SDS were added, mixed well and incubated at 50°C for a minimum of three hours.

To the digested sample, 300 μl of 5 M NaCl was added and mixed by vortexing. An equal volume of phenol (pH 7.8) saturated with Tris-HCl, was added, mixed thoroughly by inversion of the tubes for 10 minutes and centrifuged at 3500 rpm for 15 minutes. The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added. The contents were mixed thoroughly by inversion for 10 minutes and centrifuged at 3500 rpm for 15 minutes. To the aqueous phase collected in fresh tubes, equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged at 3500 rpm for 15 minutes. The supernatant was transferred to a sterile 50 ml beaker and $1/10^{th}$ volume of 3 M Sodium acetate (pH 5.5) was added and mixed. An equal volume of chilled isopropyl alcohol was added and the

precipitated DNA was spooled out using a fresh micropipette tip, washed in 70 per cent ethanol and air-dried. Dried DNA was resuspended in 0.5 ml of Tris buffer (TE-10m*M* Tris, 0.1 m*M* EDTA) and stored at –20°C.

3.4 DETERMINATION OF YIELD AND PURITY OF DNA:

The concentration of DNA samples was determined by measuring the absorbance of an aliquot of 1/100 dilution in a spectrophotometer at 260 and 280nm. The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provided an estimate of the purity of DNA. Pure preparations of DNA have an OD_{260} / OD_{280} value of 1.8. If contaminated with protein the value is lower. Quality of the DNA was also assessed on an agarose gel. If a single high molecular weight band was visualized following electrophoresis, the DNA extracted was assumed to be intact and not degraded, while a streak revealed that the DNA had been physically sheared. A staining in the gel slots indicated protein contamination.

An OD of one at 260nm wavelength corresponded to approximately 50µg/ml of double stranded DNA. Concentration of DNA stock solution was calculated as follows.

Concentration of DNA stock solution (µg/ml) = OD_{260} X Dilution factor X 50

The total yield of DNA obtained from blood was calculated by multiplying the concentration with the volume of DNA stock solution.

3.5 PCR ANALYSIS

3.5.1 Template DNA

Working solution of DNA samples were prepared from the stock solution of DNA by diluting with sterile triple distilled water to get a final concentration of 50ng/µl. One microlitre of this working solution was used in every 10µl PCR assay.

3.5.2 Selection of Primers

A set of twenty microsatellite markers were selected from available literature and database based on the polymorphicity and ten were custom synthesized. Five markers viz. S0005, S0101, SW1026, SW2517 and S0008 were chosen for the study based on the ease in typing and the polymorphicity. The sequences of the forward and reverse primers for the selected loci were as follows.

Locus		Primer Sequence (5'-3')
1. **S0005**	F	TCCTTCCCTCCTGGTAACTA
	R	GCACTTCCTGATTCTGGGTA
2. **S0008**	F	GAGGCAGTGTGTTCTATTCA
	R	GCCATGTGTAAAGTGTTGCT
3. **SW1026**	F	TGGAGAGGCAATGCTGTATG
	R	GTATTTCACCTGCAGCTCCC
4. **S0101**	F	GAATGCAAAGAGTTCAGTGTAGG
	R	GTCTCCCTCACACTTACCGCAG
5. **SW2517**	F	ATACTATGTGCTTGCGTGCG
	R	AAGGAACCCATGAGAGTACTGG

The primers obtained in lyophilized form were reconstituted with sterile triple distilled water to make a stock solution of 200pM (pico Moles per microlitre) concentration. The solutions were incubated at room temperature for one hour and then stored at -20°C. Working solutions of the primers (10pM/µl) were prepared by dilution from the stock solution.

3.5.3 End labeling of PCR primer:

For use in the PCR, one member of each pair of primer was polynucleotide kinase labelled at its 5' end with γ^{32} P ATP. The reaction was carried out in a 10µl volume containing 1µl of 10X PNK buffer (700mM Tris, 100mM $MgCl_2$, 50mM DTT), 1.6 mM primer, 6 units of PNK, 0.008 mCi of γ^{32} P ATP and 6.8µl of water and incubated at 37°C for 30 min. For setting up the PCR, the end-labelled primer was diluted with 30µl of water and 1µl of the diluted end labeled primer was used per reaction.

3.5.4 Sequencing M13 bacteriophage DNA:

Determination of the exact size of alleles necessitated comparison with a sequencing ladder from M13. Single stranded M13 phage DNA was sequenced using the DNA Sequencing Kit Version 2.0 (Amersham Biosciences Corporation, USA) according to the manufacturer's instructions as follows.

1. Preparation of annealing mixture

The composition of the mixture was as follows:

M13 phage DNA (0.2µg/µl)	-	5µl
5 X Sequenase reaction buffer	-	2µl
Forward primer (5pM/µl)	-	1µl

The volume was made up to 10µl with distilled water. The mixture was centrifuged briefly and was incubated at 65°C for two minutes. It was then slowly cooled to room temperature over 15-30 min, and then chilled on ice.

2. To the four tubes labeled G, A, T and C added 2.5 µl of each dideoxy termination mixtures (ddGTP, ddATP, ddTTP and ddCTP).

3. Dilution of labeling mix

The labeling mix provided in the kit was diluted five fold as follows:

5 X Labelling mix	-	0.5µl
Water	-	2.0µl

4. Dilution of enzyme

The enzyme (Sequenase version 2.0) was diluted eight fold with Sequenase enzyme dilution buffer as follows:

Sequenase enzyme (13U/µl)	-	0.5µl
Sequenase dilution buffer	-	3.5µl

5. Labeling reaction

To the ice cold annealed DNA mixture, the following components were added.

Dithiothreitol (DTT 0.1M)	-	1µl
Diluted labeling mix	-	2µl
Diluted enzyme	-	2µl
α^{35}S dATP (10µ Ci/µl)	-	2µl

The contents were mixed well and incubated at room temperature for two to five minutes.

6. To each termination tubes (G, A, T, C), 3.5 µl of labeling reaction mixture was transferred, mixed well and incubated at 37°C for five minutes.

7. The reaction was stopped by adding 4µl of stop solution provided in the sequencing kit and stored at –20°C.

3.6 ELECTROPHORESIS

3.6.1 Agarose Gel Electrophoresis:

The agarose gels were prepared in the horizontal plastic tray of the electrophoresis apparatus by sealing the open ends with autoclave tape to form the mould. The concentration of agarose used ranged from less than 1% to 2% for electrophoresing larger and smaller DNA fragments respectively.

One gram of agarose (Molecular Biology grade) were mixed with 100ml of 1X TBE (45 mM Tris-borate, 1mM EDTA) and heated in a microwave oven until the agarose was dissolved. The solution was cooled to 60°C, mixed with ethidium bromide (10mg/ml in water) to a final concentration of 0.5µg/ml and poured on to the mould to a thickness of 3-5mm. The combs were placed in the slot avoiding any, air bubbles, and withdrawn after the gel was set and positioned for electrophoresis.

The gel was placed in a horizontal tank with one litre of 1X TBE sufficient just to cover it. An aliquot of 10µl of the DNA sample was mixed with 5µl of loading buffer (50mM Tris, 5mM EDTA, 50% glycerol, 0.1% Bromophenol Blue) and was loaded into the wells of the gel. Electrophoresis was done for 1hr at 100 volts. The gel was placed on a transilluminator to view the products.

3.6.2 Denaturing Polyacrylamide Gel Electrophoresis

The radioactively labeled PCR products were subjected to electrophoresis on six percent denaturing polyacrylamide gels for better resolution. Denaturing Polyacrylamide Gel Electrophoresis (PAGE) was performed on the manual sequencer (Consort, Belgium) as described by Biggin *et al.* (1983). The gels were set between two glass plates (41 x 33cm) separated by 0.35 mm thick spacers.

3.6.2.1 Casting the Gel

The glass plates were cleaned thoroughly with soap solution and dried. Traces of grease and oil were removed by repeated wiping with alcohol. One of the plates was gently coated with Dimethyl dicholorosilane solution (BDH) to prevent the gel from adhering to both the plates. The plates were assembled with 0.35mm thick spacers in between and the sides and bottom sealed with sealing tape.

The gel was prepared by mixing 60ml of 0.5X TBE gel mix (6% acrylamide, 6*M* urea, 0.5X TBE) and 125µl each of 10% Ammonium persulphate solution (APS) and N', N', N', N', Tetra Methyl Ethylene Diamine (TEMED) in a beaker. The mixture was poured between the glass plates avoiding air bubbles. The plates were clamped and the comb (Shark toothed comb) inserted on top with the toothed surface facing upwards. The gel was allowed to set for an hour before electrophoresis. The tapes and clamps were removed, plates cleaned and assembled in the sequencer. The upper and lower electrode tanks were filled with 1X TBE buffer (pH 8.3) to the required level. The comb was removed, wells cleaned with buffer solution and comb was then reinserted in opposite direction with the toothed surface now facing downwards to form sample-loading wells.

3.6.2.2 Loading of Samples

The PCR products were mixed with 4.0µl formamide loading buffer (0.02% Xylene Cyanol, 0.02% Bromophenol Blue, 10m*M* EDTA, 98% deionised formamide), denatured at 95°C for 5 min and cooled immediately on ice. About 4µl each of this mixture was loaded into each well with great care to avoid mixing up of the samples from adjacent wells. Sequenced products of M13 DNA which were also denatured at 95°C for 5 minutes were loaded simultaneously in the middle or side wells.

3.6.2.3 Electrophoresis

The gels were electrophoresed at 40W for three hours maintaining a temperature of around 50°C. The bromophenol dye in the loading buffer acted as indicator of the mobility of DNA fragments and had a mobility equivalent to a 25 base fragment and the Xylene cyanol dye had a mobility equivalent to an approximately 100 base fragment.

3.6.2.4 Drying of gels

After electrophoresis the glass plates were removed from the sequencer, and carefully separated. The gel adhering to one of the plates was transferred to a filter paper. The position of the first well was marked by cutting out a small portion of the corresponding corner. The gel was covered with a Klin Wrap and dried in a gel drier at 80°C for one and a half hours.

3.6.2.5 Autoradiography

The Klin Wrap was removed after drying and the gel was set for autoradiography with X-ray film (Kodak, 35.6 x 43.2cm) in a cassette (Kiran

Hypercassette) fitted with an intensifying screen. The X-ray film was developed after 24 to 48 hours depending on the intensity of radioactive signal.

3.6.2.6 Developing X-ray film

The X-Ray film was developed in the dark room. Developing was done by transferring the film serially into the developer solution (Kodak) for three to five minutes, 1% acetic acid for a minute followed by washing in distilled water and finally into fixer solution (Kodak) for six to ten minutes. The developed film was washed thoroughly in running water and dried.

3.6.2.7 Microsatellite typing

The genotypes of the animals were determined for each microsatellite loci by comparing the sizes of alleles with M13 sequencing ladder. The G, A, T and C sequences were read from the bottom to the top in order. The allele sizes were assigned corresponding to the G, A, T, C bands. The frequency at each locus was determined by direct counting.

3.7 STATISTICAL ANALYSIS

3.7.1 Allele Frequency

The allele frequencies of various markers were checked, and the markers with maximum frequency were selected for analysis. Since very few markers were tested for allele frequency in indigenous breeds, the available frequencies were considered for testing in native pigs also.

Heterozygosity, Polymorphic Information Content (PIC), number of alleles and allele size range were worked out for each locus and for each population studied.

3.7.2 Heterozygosity (He)

Heterozygosity is a measure of usefulness of the marker. Heterozygosity was calculated by the method of Ott (1992).

Heterozygosity is given by;

$$He = 1 - \sum_{i=1}^{k} P_i^2$$

Where p_i is the frequency of i^{th} allele at a locus. Markers with higher heterozygosity values are more useful.

3.7.3 Polymorphic Information Content (PIC)

The polymorphic information content expresses informativeness or usefulness of a marker for linkage studies. The PIC values of the markers were calculated as;

$$PIC = 1 - [\sum_{i=1}^{k} p_i^2] - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2p_i^2 p_j^2$$

Where p_i and p_j are the frequencies of i^{th} and j^{th} alleles, respectively (Botstein et al, 1980).

3.7.4 Phylogenetic Analysis

The computation of genetic distance and the phylogenetic analysis were done using the programs in the POPGENE package version 1.32. The programs used in the analysis were:

Genetic distance: Genetic distance measures provide the best description of genetic differentiation among populations or genetic groups. The standard genetic distance between the four populations studied were computed using Nei's method as given by Nei (1978). This programme computes the genetic distance matrix from a set of gene frequencies in different populations (or species). Nei's standard genetic distance is given by;

$$Ds = -\ln [J_{XY} / \sqrt{J_X J_Y}]$$

where J_X, J_Y and J_{XY} are averages of $\sum x_i^2$, $\sum y_i^2$ and $\sum x_i y_i$ over r loci and x_i and y_i are the sample allele frequencies of the i^{th} allele in populations X and Y, respectively.

Dendrogram: A dendrogam representing the relationships of the animals belonging to the populations was constructed using the POPGENE version 3.2. The UPGMA method was used for plotting the dendrogram.

Results

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

A total of 100 DNA samples were isolated from whole blood obtained from the various genetic groups of pigs, all over the Kerala. The morphological features of different groups were documented before sample collection. The samples were divided into four different populations based on the area from where samples were collected.

4.1.1 Yield and Quality of DNA

The mean concentration and yield of DNA obtained from whole blood was 448.69±51.43µg/ml and 224.35±9.86µg/5ml, respectively. The average value of the ratio of optical density at 260 and 280 nm, which indicate the purity of DNA isolated, was found to be 1.68±0.016. The values are presented in Table 4.1.

The DNA samples were checked for quality in 1.5% agarose gel. On electrophoresis single bands without smears were observed indicating that there were no sheared fragments and that the DNA collected was of good quality.

4.2 PCR ANALYSIS

Porcine microsatellite primers selected from database and published literature, were custom synthesized and used for PCR amplification of the samples. Out of the 20 markers tested, most markers exhibited very low or moderate degree of polymorphism in native pigs. Based on the polymorphicity and ease in typing five markers were selected for study, viz. S0005, S0101, SW1026, SW2517 and S0008. PCR amplification was performed on all the 100 samples using the primer pairs for the five selected loci.

4.2.1 Optimization of PCR

The conditions of PCR, temperature and time of the cycles were optimized separately for each primer for the efficient amplification. The reaction conditions and cycle parameters for each primer pair is presented in Tables 4.2 and 4.3. The PCR products were checked for amplification on 1.5% agarose gels using *Hae*III digested pBR322 as the size standard. Amplified products of the expected size range were observed for all selected loci. The amplified PCR products of the marker S0005 checked on agarose gel is shown in plate.3.

4.3 NUMBER OF ALLELES, SIZE AND FREQUENCY

The amplified products were fractionated by denaturing polyacrylamide gel electrophoresis. The pattern of bands produced at each locus was visualized by autoradiography. Direct counting method was employed for detecting the number of alleles at each locus. The allele sizes were checked by comparing with sequence of M13 mp18 single standard DNA, which was run along with the samples. Among the five loci studied, the marker S0008 showed the presence of shadow bands. The different genotypes and their frequencies obtained for the selected loci in various genetic groups are presented in Tables. The different alleles and their frequencies for the five markers are presented in Tables 4.4 to 4.8. The allele size, range, number of alleles, heterozygosity and PIC for the different loci tested is summarized in tables 4.9 to 4.13.

4.3.1 S0005

Eight alleles ranging in size from 202-240 bp were detected in indigenous pigs. All the eight alleles were present in population I, but only seven alleles were noted in population II, the allele size of 240 was not seen in population II, III and IV. One more allele of size 236 found in population I and II only. The alleles and their

frequencies for this locus are presented in Table 4.4. The polymorphic pattern of this marker is shown in plate 4.

4.3.2 S0101

In the four population studied, eight alleles were noted in the size range of 204-222 bp. Seven alleles have been seen in population III, when only three alleles, viz. 204,206,210 were seen in population I and these alleles were not present in population IV. The allele 206 was found in all populations but population III. The other groups had fewer alleles, five in population IV, four in population II. The results are presented in Table 4.5. The autoradiograph of the gel with the amplified products of the marker S0101 is shown in plate5.

4.3.3 SW1026

Five alleles were detected for SW1026 locus in the pooled population. All the five alleles ranging from 98-120 were present in population I, III and IV. However two alleles viz. 110 and 120 were not noted in population III. Allele frequency and size of alleles for this locus is presented in Table 4.6. Plate 6 reveals the polymorphic pattern of the marker S1026.

4.3.4 SW2517

This marker is comparatively more polymorphic with six alleles size ranging from 146-178 in the pig population analysed. The first population from university pig farm contained all six alleles. The second population contained only three alleles with the sizes of 146, 150 and 160. The third and fourth population contained four alleles each. The alleles with the size of 178,166 were not found in these two populations. The different alleles and their frequencies obtained in the four populations are presented in Table 4.7. the results obtained at the end are presented in plate 7.

4.3.5 S0008

Five alleles were detected for this locus with the allele size ranging from 172-192 bp. The allele with the size of 184 is missing in population III and the allele size of 192 is not found in population III. The amplification of this locus in the first two populations was poor, however all five alleles were present in third population and four alleles were detected in the fourth population. The alleles and their frequencies are presented in Table 4.8. Plate 8 shows the alleles of marker S0008 and their polymorphism.

4.4 HETEROZYGOSITY

The heterozygosity values for each locus were found out by the method suggested by Ott (1992). The heterozygosity values for each marker in different population are presented in Tables 4.9 to 4.13. The mean values obtained were 0.8475 for S0005, 0.7775 for S0101, 0.7672 for SW1026, SW2517 and 0.7424 for S0008. The mean values are presented in Table 4.14.

4.5 POLYMORPHIC INFORMATION CONTENT

Polymorphic information content is a measure of the informativeness of a marker and was calculated using the method of Botstein *et al.* (1980). The PIC values for each marker in the different breeds are presented in Tables 4.9 to 4.13. The mean PIC values obtained were 0.8291 for S0005, 0.7483 for S0101, 0.7284 for SW1026, 0.7381 for SW2517 and 0.6974 for S0008. The mean values are presented in Table 4.14.

4.6 GENETIC DISTANCE

The genetic distances among population were calculated according to Nei's formula (Nei, 1972), using the software POPGENE version 3.2. The genetic distance

between population II and IV is maximum, 0.7161. Nei's genetic distance between I and II population is 0.6754, between population III and I is 0.4818, between III and II is 0.6121, between I and IV is 0.4898 and between population III and IV is 0.5704. The genetic distances are presented in Table 4.15.

4.7 DENDROGRAM

Dendrogram of relationships between the four populations under study was plotted using the POPGENE program. The dendrogram showed the population I and IV form one cluster and the II and III form another.

Table 4.1 Concentration, yield and purity of DNA obtained from the blood samples of the four pig populations.

Parameter	Population-I	Population-II	Population-III	Population-IV	Average
Concentration (µg/ml)	756	510	263.2759	265.5000	448.6940
Yield (µg/0.5ml)	378	255	131.6379	132.7500	224.3470
OD ratio (260nm/ 280nm)	1.9805	1.4145	2.0706	1.2711	1.6842

Table 4.2 Standardized conditions for the PCR assay

Sl. No.	Parameter	Value
1.	Template DNA (ng)	50
2.	$MgCl_2$ (m*M*)	1.25
3.	10X Reaction Buffer (µl)	1
4.	dNTPs (µ*M*)	200
5.	Forward Primer (p*M*)	5
6.	Reverse Primer (p*M*)	5
7.	Taq DNA Polymerase (U)	0.3
8.	Reaction Volume (µl)	10

Table 4.3 Standardized temperature and cycling conditions for the PCR at different microsatellite loci analysed.

Sl. No.	Parameter	Temperature/cycles				
		S0005	S0101	SW1026	SW2517	S0008
1.	Denaturation (°C for 1min)	94	94	94	94	94
2.	Annealing (°C for 1min)	60	60	58	58	58
3.	Extension (°C for 1min)	72	72	72	72	72
4.	No. of Cycles	35	35	35	35	35
5.	Final extension (°C for 5 min)	72	72	72	72	72

Table 4.4 Alleles and their frequency at the S0005 locus in the four pig populations.

Allele (bp)	Frequency				
	Population I	Population II	Population III	Population IV	Pooled population
1 (202)	0.0735	0.0833	0.4310	0.3000	0.2342
2 (208)	0.0147	0.0833	0.1379	0.1500	0.0823
3 (214)	0.1618	0.0833	0.0862	0.1000	0.1203
4 (220)	0.1029	0.0833	0.2241	0.1500	0.1519
5 (224)	0.1324	0.2500	0.1034	0.1500	0.1329
6 (230)	0.2647	0.2500	0.0172	0.1500	0.1582
7 (236)	0.2059	0.1667	0.0000	0.0000	0.1013
8 (240)	0.0441	0.0000	0.0000	0.0000	0.0190

Table 4.5 Alleles and their Frequency at the S0101 locus in the four Pig Populations.

Allele	Frequency				
	Population I	Population II	Population III	Population IV	Pooled population
1 (204)	0.7206	0.5000	0.0345	0.0000	0.3636
2 (206)	0.0147	0.0000	0.0690	0.0000	0.0325
3 (210)	0.2647	0.3000	0.1034	0.0000	0.1753
4 (212)	0.0000	0.1000	0.3793	0.5000	0.2078
5 (214)	0.0000	0.0000	0.0862	0.1667	0.0519
6 (216)	0.0000	0.0000	0.2241	0.0556	0.0909
7 (218)	0.0000	0.1000	0.1034	0.1667	0.0649
8 (222)	0.0000	0.1000	0.0000	0.1111	0.0130

Table 4.6 Alleles and their frequency at the SW1026 locus in the four pig populations.

Allele	Frequency				
	Population I	Population II	Population III	Population IV	Pooled population
1 (98)	0.2647	0.3333	0.1481	0.1000	0.2133
2 (106)	0.1471	0.5833	0.3889	0.5500	0.3133
3 (110)	0.2353	0.0000	0.2222	0.2000	0.2000
4 (116)	0.2794	0.0833	0.1852	0.1000	0.2133
5 (120)	0.0735	0.0000	0.0556	0.0500	0.0600

Table 4.7 Alleles and their frequency at the SW2517 locus in the four pig populations.

Allele	Frequency				
	Population I	Population II	Population III	Population IV	Pooled population
1 (146)	0.2813	0.2000	0.2857	0.3333	0.2838
2 (150)	0.2969	0.0000	0.2500	0.3889	0.2703
3 (154)	0.0938	0.2000	0.3393	0.2222	0.2095
4 (160)	0.2031	0.0000	0.1250	0.0556	0.1419
5 (166)	0.1094	0.6000	0.0000	0.0000	0.0878
6 (178)	0.0156	0.0000	0.0000	0.0000	0.0068

Table 4.8 Alleles and their frequency at the S0008 locus in the four pig populations.

Allele	Frequency				
	Population I	Population II	Population III	Population IV	Pooled populatoin
1 (172)	0.1795	0.1890	0.1923	0.1667	0.1857
2 (180)	0.2478	0.3495	0.3846	0.1111	0.3143
3 (184)	0.1843	0.0000	0.2115	0.0000	0.1571
4 (188)	0.4113	0.2629	0.2115	0.6111	0.3143
5 (192)	0.0000	0.0699	0.0000	0.1111	0.0286

Table 4.9 Allele size range, Number of alleles, Size range, Heterozygosity and Polymorphic Information Content (PIC) at the S0005 locus in the four pig populations.

Observation	Population I	Population II	Population III	Population IV
No. of alleles Size range (bp)	8 (202-240)	7 (202-236)	6 (202-230)	6 (202-230)
Heterozygosity	0.8257	0.8194	0.7265	0.8100
PIC	0.8031	0.7956	0.6893	0.7841

Table 4.10 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the S0101 locus in the four pig populations.

Observation	Population I	Population II	Population III	Population IV
No. of alleles Size range (bp)	3 (204-210)	5 (204-222)	7 (204-218)	5 (212-222)
Heterozygosity	0.4105	0.6400	0.7711	0.6790
PIC	0.3374	0.5812	0.7422	0.6402

Table 4.11 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the SW1026 locus in the four pig populations.

Observation	Population I	Population II	Population III	Population IV
No. of alleles Size range (bp)	5 (98-120)	3 (98-116)	5 (98-120)	5 (98-120)
Heterozygosity	0.7695	0.5417	0.7401	0.6350
PIC	0.7309	0.4598	0.6995	0.5951

Table 4.12 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the SW2517 locus in the four pig populations.

Observation	Population I	Population II	Population III	Population IV
No. of alleles Size range (bp)	6 (146-178)	3 (146-166)	4 (146-160)	4 (146-178)
Heterozygosity	0.7705	0.5600	0.7251	0.6852
PIC	0.7338	0.4992	0.6736	0.6237

Table 4.13 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the S0008 locus in the four pig populations.

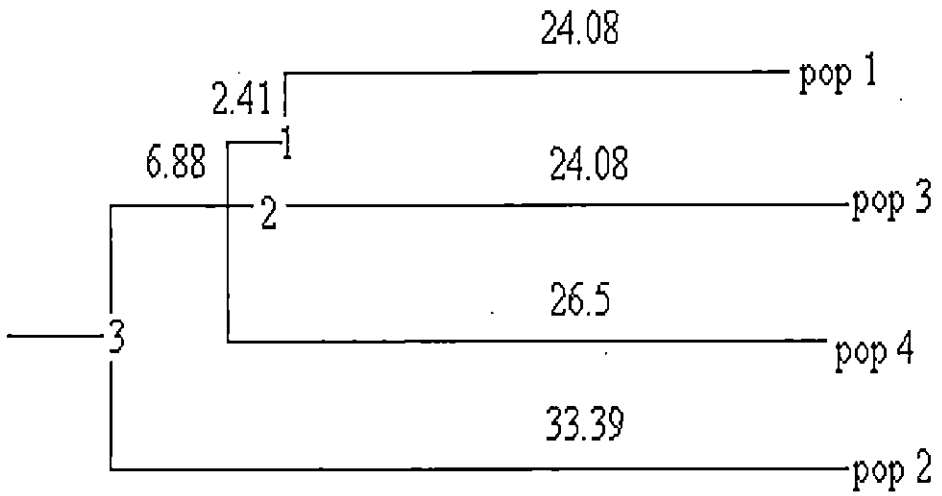
Observation	Population I	Population II	Population III	Population IV
No. of alleles Size range (bp)	4 (172-188)	4 (172-192)	4 (172-188)	4 (172-192)
Heterozygosity	0.6865	0.7426	0.7988	0.5741
PIC	0.6519	0.7113	0.7706	0.5332

Table 4.14 Mean Heterozygosity and PIC of the five microsatellite markers tested in the different pig populations.

Parameter	Microsatellite loci				
	S0005	**S0101**	**SW1026**	**SW2517**	**S0008**
Mean Heterozygosity	0.8475	0.7774	0.7672	0.7747	0.7424
Mean PIC	0.8291	0.7483	0.7284	0.7381	0.6974

Table 4.15 Nei's Genetic distance matrix for the four pig populations analysed.

	Population I	**Population II**	**Population III**	**Population IV**
Population I	***			
Population II	0.6754	***		
Population III	0.4818	0.6121	***	
Population IV	0.4898	0.7161	0.5704	***



Dendrogram based Nei's (1972) genetic distance drawn by UPGMA method.

Plate 1. Indigenous pig of Kerala- Hairy with short snout and dished back

Plate 2. Indigenous pig of Kerala- with long snout and non- dished back

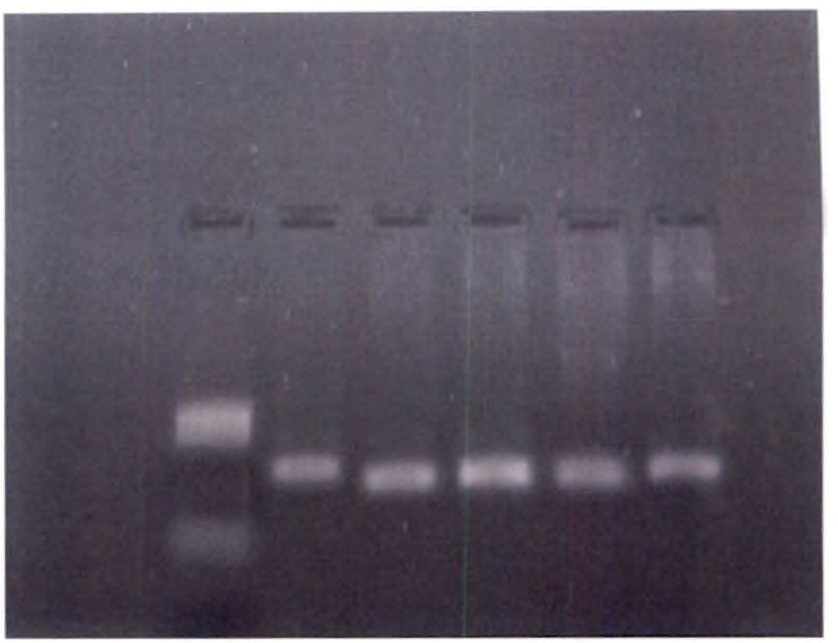


Plate 3. Agarose gel electrophoresis - S0005
First lane - pBR322 *HaeIII* digest as marker

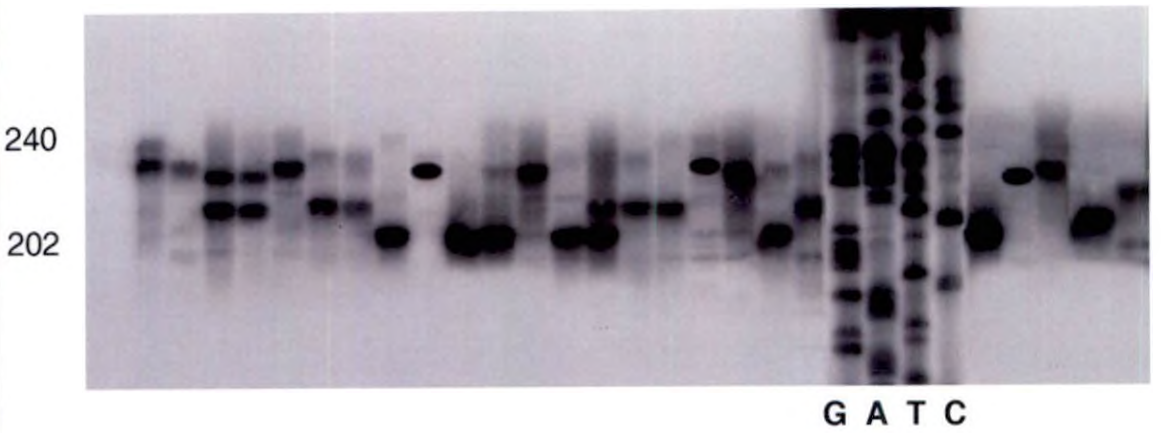


Plate 4. Autoradiograph showing polymorphism at S0005 locus GATC represents M13 sequence used as marker

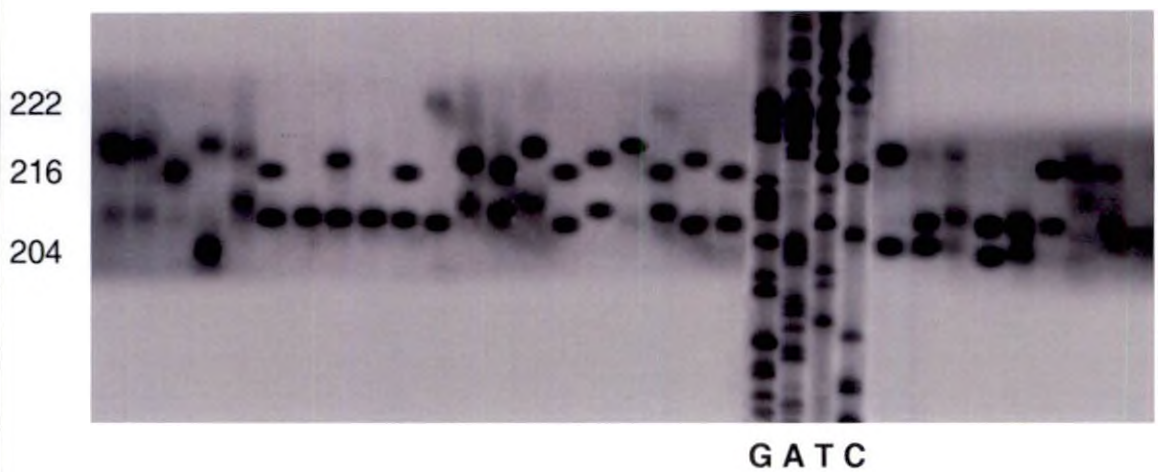


Plate 5. Autoradiograph showing polymorphism at S0101 locus GATC represents M13 sequence used as marker

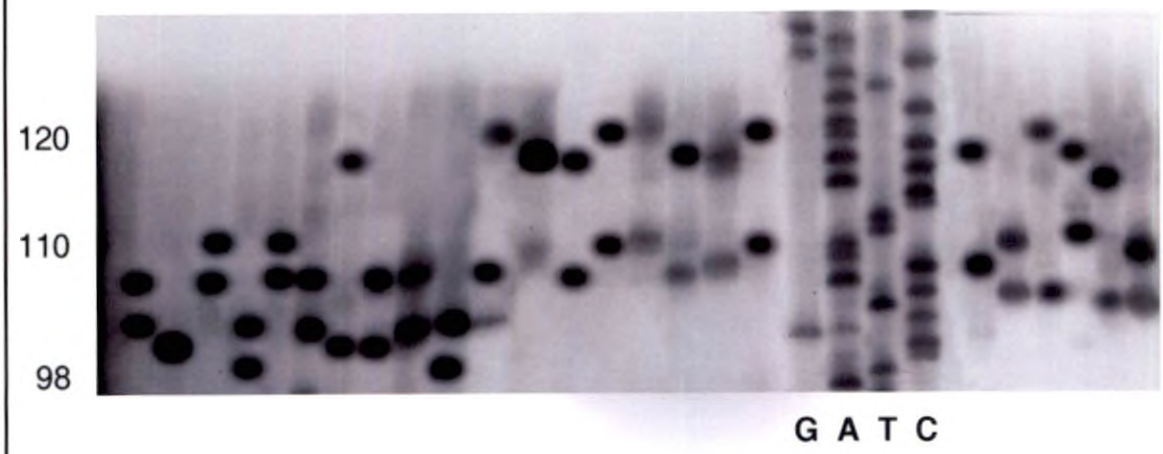


Plate 6. Autoradiograph showing polymorphism at SW1026 locus GATC represents M13 sequence used as marker

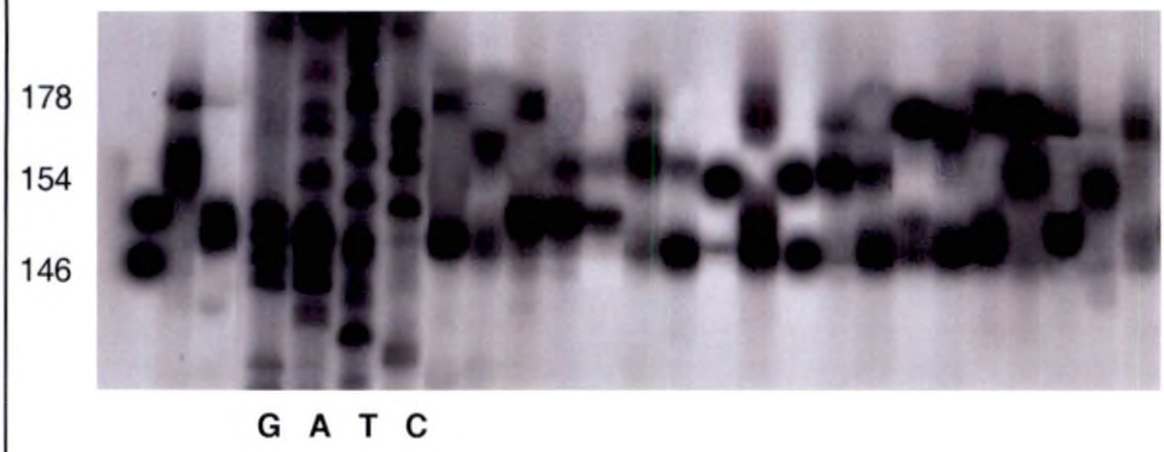


Plate 7. Autoradiograph showing polymorphism at SW2517 locus GATC represents M13 sequence used as marker

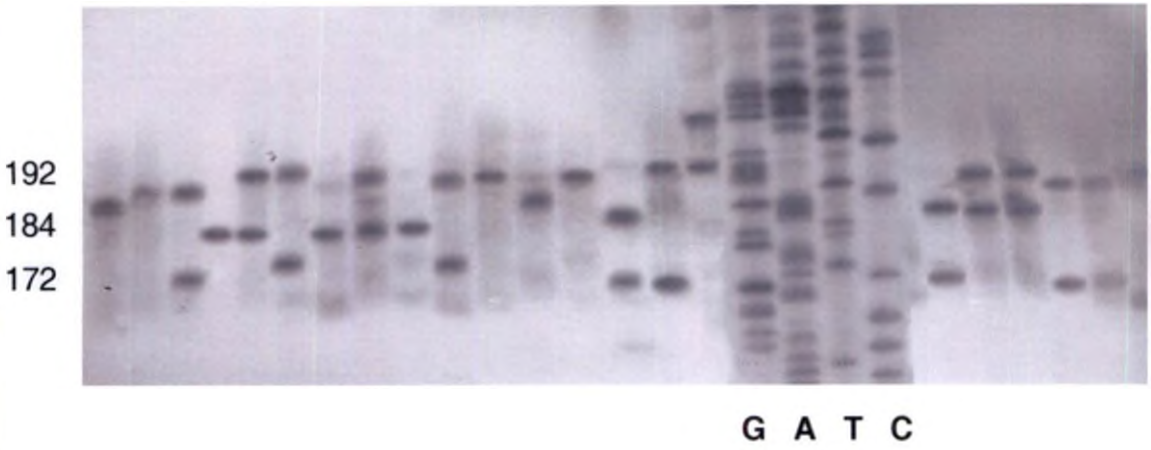


Plate 8. Autoradiograph showing polymorphism at S0008 locus GATC represents M13 sequence used as marker

Discussion

5. DISCUSSION

The pigs present through out the world are different from each other in morphology and production traits. There are 600 breeds of pigs in the world (Giuffra *et al.*, 2000). The pigs found in different countries are genetically diverse and show phenotypically distinct characters, eg: Mexican hairless pigs and Chinese Meishan pigs. In India many of such native breeds exist in small population size, almost in the verge of extinction. These native breeds are evolved by nature and are resistant to various diseases and climatic extremities. A systemic evaluation of genetic diversity of these important breeds will enable us to understand these breeds better and formulate plans to conserve them. Microsatellite markers were proved to be useful for the analysis of genetic relationship and characterization (Weber, 1990; Kemp and Teale, 1991). This study was conducted with the objective of analyzing the genetic relationship among the indigenous pigs of Kerala and characterizing them into different groups.

5.1 SOURCE OF DNA:

Although all the local pigs available in central Kerala are called as Anagamali pigs, phenotypically distinct populations exist within them. Some animals have short snouts and possess dished back and some have long snouts and do not possess dished back. Some are less hairy and has round barreled stomach and some are hairy and pot bellied. A small population of about forty Angamali pigs were conserved and maintained in Centre for Pig Production and Research, Mannuthy. Blood samples were collected from these animals for the study. Blood samples were collected from different parts of Kerala including Koothattukulam and border districts of Kerala. Various slaughter houses were also approached for blood collection. Samples

were collected after noting their phenotypic characters. The blood was collected from ear vein in older pigs and anterior vena cava in pigs of less than six months age.

5.2 ISOLATION AND YIELD OF DNA:

Isolation of genomic DNA was performed following Phenol-Chloroform method as described by Sambrook *et al.* (1989). This was easy to adopt and efficient technique for isolation of DNA from blood.

The mean yield of DNA from 5 ml of blood was 224.35±9.86 μg. Pigs have maximum leukocytes per ml of blood in domestic species, 11.0 – 2.0 X 10^3. However Andersson *et al.* (1986) and Aravindakshan *et al.* (1998) reported yields of 200.35±21.54 μg and 394.50±34.26 μg respectively per 10-15ml of cattle blood which have leukocyte count of 5.5 – 19.5 X 10^3. The higher yield in pigs may be due to comparatively higher leukocytes per unit volume of blood. Senthil *et al.* (1996) reported as high as 444.58±21.5 μg/10 ml of blood.

5.3 MICROSATELLITE ANALYSIS:

5.3.1 PCR Conditions:

Polymerase chain reaction involves *in vitro* enzymatic synthesis of millions of copies of desired DNA fragment. In this method two oligonucleotide primers that flank the template DNA anneal after heat denaturation of DNA followed by extension of annealed primers with the help of DNA polymerase. This result in the exponential accumulation of the specific target fragment approximately 2^n times, where n is the number of cycles (Saiki et *al.*, 1988).

Various PCR conditions were tested and optimized for the selected markers to obtain specific products. Magnesium chloride concentration and annealing temperature of the primer were critical in obtaining specific amplifications.

The annealing temperature for the primer pair was optimized using gradient thermal cycler in the range of 56-64°C. An annealing temperature of 60°C was found optimal for the primers S0005 and S0101. This is in concordance with Fredholm *et al.* (1993) and Rohrer *et al.*, (1996). The markers SW1026, SW2517 and S0008 yielded good products at 58°C though the recommended annealing temperature was 60°C (Mikawa *et al.*, 1999; Alexander *et al.*, 1996; Rohrer *et al.*, 1994). Although the magnesium chloride concentration recommended was 1.5 *mM,* (Andersson *et al.*, 1994; Coppieters *et al.*, 1993; Ellegren *et al.*, 1993) the primers yielded specific products at 1.25 m*M* concentration.

Other conditions affecting the specificity of the polymerase chain reaction include concentrations of template DNA, enzyme, primer, the annealing time, extension time and number of cycles. All these conditions except the DNA concentration was kept constant for all the primers used. A concentration of 0.3 U/reaction of *Thermus aquaticus* DNA polymerase and 5 p*M* of primers per reaction gave satisfactory results. Time for denaturation, annealing and extension was one minute with a final extension time of five minutes.

5.3.2 Microsatellite Primers:

Primers were selected from the database and available literature based on their allele frequency and heterozygosity. About 20 highly polymorphic primers were selected from all over the genome (Rhorer *et al.* 1994). These

primers were custom synthesized and tested in indigenous population. Some primers were not polymorphic and some yielded products but were difficult to analyse. Most of the primers were only moderately polymorphic in the population studied. Among these primers, five markers that were found most polymorphic, and were comparatively easy to analyse with sharp bands were selected for further analysis. The markers selected for this study were S0005, S0101, SW1026, SW2517 and S0008.

5.3.2.1 S0005:

This marker was found to be highly polymorphic and informative. Eleven alleles were reported by Fredholm *et al.* (1993), Rhorer *et al.* (1996) with the size ranging from 194-248 bp. Eight alleles were observed in this study among the four pig populations. All the eight alleles were observed in population I followed by seven in population II and six in population III and IV. The observed allele size range varied between 202 – 240 bp. The allele size of 240 bp was found only in population I and the allele size 236 bp was observed in population I and II. This marker was found fairly polymorphic in indigenous pig population and strongly recommended for characterization purpose by Kaul *et al.* (2001). Observations in this study coincide with other results in indigenous pigs, of North Eastern India with the suggestion that this marker can be useful for characterization of native pigs.

5.3.2.2 S0101:

A total of eight alleles were detected for this locus in the pooled population. Among these the smallest three alleles of size 204 – 210 bp were noticed in population I and only the biggest five alleles of size 212 –222 bp were seen in population IV. Thirteen alleles were detected at this locus in American pig population by Rhorer *et al.*, 1996. Groenen *et al.* (2003)

reported this marker to be less polymorphic but fairly useful for chracterisation studies, and recorded six alleles in French pig population. Decrease in number of alleles in population I and IV could be because of inbreeding and hence less genetic variation existed between animals.

5.3.2.3 SW1026:

Five alleles were observed at this locus among the four pig populations. All four alleles were observed in population I, III and IV. However only three alleles were reported in population II. The size range of alleles varied between 98 and 120 bp and the alleles of size 110 bp and 120 bp were absent in population II. This size range reported in the present study is same as reported by Rhorer *et al.*, 1996. Even though this marker yielded only five alleles in indigenous pig population of Kerala, 15 alleles were observed in European breeds (Rhorer *et al.*, 1996). This may be due to the population size of indigenous pigs are very small and are inbred for many generations. In the field conditions only one to two males were maintained per 30-40 females for many years. However the allele showed significant variation between populations.

5.3.2.4 SW2517:

This marker was found to be the moderately polymorphic among the five markers typed with six alleles in the size range between 146-178 bp. All six alleles were observed in population I. Only three alleles were seen in population IV while four alleles were observed in population III and IV. An average allele size of 172 bp was reported in pigs by Alexander *et al.* (1996). The marker contains (AC)n dinucleotide repeats and maximum of 29 alleles were reported by Rhorer *et al.* (1996). The indigenous pig population showed less number of alleles would be attributed to nuclear population and

rampant inbreeding. Similar results were reported by Li *et al.* (2003). More number of alleles was detected in exotic population, because they were distinct and would have had a wider genetic base. Since this marker is linked with the genetic disorder anal atresia (Pamela *et al.*, 2005), the result showed the least possibility of the disease in indigenous pig population.

5.3.2.5 S0008:

Though total of five alleles were detected at this locus in the pooled population, only four alleles were present in each of the population II, III and IV. The size of alleles ranged from 172-192 bp. Same number of alleles was detected at this locus in European pig population by Mikawa *et al.* (1999). Fifteen alleles have been reported by Fredholm *et al.*(1993) in exotic population. Same number of alleles were observed by Rhorer *et al.* (1996) and Lopez *et al.* (1999) in different exotic populations. The presence of shadow bands was a feature noticed for this primer. According to Murray *et al.* (1993) shadow bands were produced during PCR with DNA sequences containing a CG dinucleotide repeat due to two base pair deletions occurring randomly in the CG repeat region. Other reasons attributed for the production of shadow bands were due to slippage synthesis or recombination events.

5.4 ALLELE FREQUENCY:

The number of alleles at different marker loci and their frequencies are indicators of genetic variability and thus form the basis of all diversity indices for estimation of genetic distances and construction of phylogenetic trees.

Those loci which were polymorphic and had at least five alleles in the population studied were selected. Ease of typing was also considered while selecting the primers. Number of alleles noted in the population studied were less than that observed in most of European and Chinese breeds (Rhorer *et al.*, 1996; Yang *et al.*, 2003), however this is in concordance with the work done in other Indian breeds (Kaul *et al.*, 2001). Among the five markers S0005 had eight alleles in population I, and six alleles in population III. This showed that the genetic diversity existed in these two populations. Less number of alleles, were detected in population I and IV for the marker S0101. Similar results of within breed genetic variation were reported by Wang *et al.* (2004) in China and Yang *et al.* (2003).

The mean number of alleles for a particular marker is dependent on sample size and the population from where the samples were taken. The number of observed alleles tends to increase withincrease in population size.

Significant difference could be detected among the populations for the allele distributions at the five loci studied. It was observed that the marker with few alleles in one population exhibited more polymorphism in other populations. Similar results were observed by Lemus *et al.* (2001). Population I showed maximum polymorphism for all loci studied. Six alleles were detected for the marker SW2517 in population I, but only four alleles were present in population III and IV for this marker. Such variations in microsatellite polymorphism have been reported in other Indian species in comparison with European breeds, Arranz *et al.* (1996) and Barker *et al.* (1991) in cattle and buffalo respectively.

The occurrence of a certain alleles with high frequency in the different populations could be suggestive of probable linkage of these loci to fitness traits and selection for those traits. However, to obtain more

consistent results and to draw definite conclusions, the study should be undertaken incorporating a higher number of animals representing more genetic groups from wider area. Another probable reason for the predominance of certain alleles in a population is the high rate of inbreeding within the populations. This is more likely due to small population size and the numbers of males in most domestic animal populations were very low.

High mutation rates account for the large variability displayed by the microsatellite markers. Irregularity of microsatellite distributions can be attributed to the complex evolution at these loci. Single step mutation events account for about 90 per cent of microsatellite mutation events, followed by double step mutations and a limited number of multi step mutations (Weber and Wong, 1993). It is suggested that the extended single step mutation produced a much larger variance of allele size when compared to the multiple step mutation model.

5.5 HETEROZYGOSITY AND POLYMORPHIC INFORMATION CONTENT:

Heterozygosity is a measure of genetic variability within a population. The heterozygosity values were found to be high for all the populations at all loci and ranged from 0.7424 to 0.8475. Takezaki and Nei (1994) determined that, for the markers to be useful for measuring genetic variation, the average heterozygosity should be between 0.3 and 0.8 in the population. The range of heterozygosity of most of the markers in populations studied was within this range and hence the markers were appropriate for measuring genetic variation. This is in concordance with the values given by Kaul *et al.* (2001). Though the heterozygosities observed in the pig populations under study is similar to the values reported in Chinese pigs (Li *et al.*, 2000), lower heterozygosity values are reported for European pig breeds (Fredholm *et al.*,

1993; Laval *et al.*, 2000). The lower heterozygosity values for European breeds may be because European breeds are synthetic and subjected for artificial selection for many generations for fixation of traits. But indigenous pig breeds are naturally evolved and are never subjected to artificial selection and so these animals have more variation within breed than European breeds.

The PIC value ranged between 0.6974 (S0008) to 0.8291 (S0005) for the five markers in the population. This suggest the suitability of these microsatellites as markers as tools for measuring genetic distance. The same range of values was reported by Kaul *et al.* (2001) in Indian pigs. The obtained values showed that the markers differed significantly among the tested populations.

5.6 GENETIC DISTANCE:

The Nei's genetic distance between populations ranged from 0.4808 to 0.7161. The genetic distance measures the time that has elapsed since the populations were genetically equivalent. In other words, it indicate the time of divergence of the breeds from each other. The allele frequencies at each locus for each population were used for computing the genetic distance between each pair of populations. According to the results obtained, the population II and IV show considerable distance (0.7161) from each other and form different clusters. The population III and IV are equally far from population I with the genetic distance of 0.4818 and 0.4898 respectively. The genetic distance of population II and IV from population III are moderate, i.e., 0.6121, 0.5704 respectively. The results show that the samples collected from Ollur and Koothattukulam form two distinct genetic pools. Further investigation is needed for their characterization purpose.

5.7 DENDROGRAM:

The dendrogram is a phylogenetic tree to show the relationship among different populations. A dendrogram was constructed from the genetic distance data using the POPGENE version 3.2. It grouped population I and IV in one cluster and population II and III in another cluster. But the length between clusters and between populations is considerably greater, which indicate that the population showed variation within and among clusters. It is a clear indication that various genetically different populations exist in the studied group.

The results from the five markers indicate that all four populations differed from each other. Even though clustering is found between groups, variation is noticed between and within populations for the different parameters tested like number of alleles, allele frequency distribution, and heterozygosity. The two morphologically distinct groups noted in this study – hairy with long snout and non-dished back were found in population I, the other – less hairy, short snout and dished back were found in population III, which were grouped under two separate clusters. In the dendrogram the lack of clarity can be attributed to the small sample size and is indicative that the populations were either inbred or formed a mixed group. More amount of variation can be noted by increasing the population size and number of markers.

It was found that the set of microsatellite markers tested in this study could be used satisfactorily for molecular characterisation and genetic diversity studies in indigenous pigs of Kerala. However, use of more number of markers and increasing the sample size would allow a better differentiation and characterization of populations.

Summary

6. SUMMARY

The alarming situation of vanishing genetic resources has incited the study of genetic diversity and characterization of native animals presuming, is the basic step for conservation. Population genetic structure within domestic breeds is an essential component of characterisation. Among various markers for characterisation microsatellites have been proved to be successful in defining genetic structure and genetic relationships among different populations. These markers display high levels of polymorphism and are consequently found to be the effective tool for differentiation and characterization. In this study, an attempt is made to study the genetic diversity and to characterize the fast disappearing species, indigenous pigs of Kerala.

The genomic DNA was isolated from 100 genetically unrelated animals of various populations using the phenol-chloroform extraction procedure. The mean yield obtained per five ml of blood was 224.35±9.86µg. The average value for the ratio of optical density (260 nm/280 nm) was 1.68±0.016.

Twenty microsatellite markers were selected from database and the published literature based on the information on polymorphism and heterozygosity. All the primers in the panel were tested and five primers chosen based on the polymorphism and ease in typing were custom synthesized and used for PCR analysis. PCR conditions were optimized separately for each marker. Each reaction was carried out in 10 µl volume and contained 1 µl of 10X PCR reaction buffer, 200 µ*M* dNTP, 5 p*M* each of forward and reverse primers and 0.3 U of the *Thermus aquaticus* DNA polymerase. $MgCl_2$ concentration of 1.25 m*M* yielded satisfactory results for all the primers. The forward primer of each marker was end labeled with γ^{32}P-ATP and the PCR products were visualized by autoradiography.

The sequenced M13 phage DNA was used as the marker for comparing alleles and the sizes. The sequencing was done by the dideoxy method following manufacturer's instructions in the Sequenase Version 2.0 (Amersham Biosciences, USA) sequencing kit.

The denaturing polyacrylamide gel electrophoresis (6% urea PAGE) was used to fractionate the amplified products. The gel after electrophoresis and drying was set for autoradiography with X-ray film (Kodak, 35.6 x 43.2 cm) in a cassette (Kiran Hypercassette) fitted with an intensifying screen. The X-ray film was developed after 12 to 48 hours depending on the intensity of radioactive signal. The number of alleles at each locus for each population was scored and size was determined by comparing with the sequence of M13mp18 single stranded DNA size standard.

A total of eight alleles were detected at the S0005 locus size ranging from 202-240 bp. The mean heterozygosity and PIC values obtained were 0.8475 and 0.8291 respectively. The S0101 locus was polymorphic with eight alleles in the size range of 204-222 bp, with a mean heterozygosity and PIC values of 0.7774 and 0.7483 respectively. Five alleles were detected at the SW1026 locus with the size range 98-120 bp. The mean values of heterozygosity and PIC were 0.7672 and 0.7284 respectively. The SW2517 marker had six alleles with the heterozygosity and PIC values of 0.7747 and 0.7381 respectively and the allele size varied from 146 to 178 bp. Amplification at the S0008 locus was not optimal and yielded only weak products of size range from 172-192 bp. Nevertheless, five alleles were detected and the mean heterozygosity and PIC values were 0.7424 and 0.6974 respectively.

Genetic distance was calculated using allele frequencies at each locus for each population. Nei's genetic distance was worked out using the Popgene program version 3.2 software package. The distance measure between population II and IV was 0.7161 which was the highest value, followed by that between population I and

II which was 0.6754. The genetic distance between population III and IV was 0.5704. The lowest genetic distance of 0.4818 was noted between population I and III. This is similar to the genetic distance between population I and IV which is 0.4898, indicating that these breeds had diverged recently when compared to other breed pairs. The dendrogram constructed using the POPGENE version 3.2 program grouped the population I and IV in one cluster and population II and III in another.

The results in the study showed variation existed among the populations for the different measures like number of alleles at each locus and heterozygosity. Animals from population I showed a higher range of allele sizes and more number of alleles at the different loci typed. These are the Angamali pigs conserved in university pig farm, which were distinct from others. The results further indicated that within population variation was more in this population and need more detailed study to investigate the same. The occurrence of wide range of alleles and the genetic distance measure indicate that the populations were different from each other.

From this study, it can be concluded that the microsatellite markers are efficient tools for molecular characterisation and genetic diversity studies in indigenous pigs. However, inclusion of more markers and increase in the sample size is recommended to enable more accurate differentiation and characterization of animals that are closely related.

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Annexure

ANNEXURE – 1

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Acrylamide (40 %)

Acrylamide	380 g
N_1N – Methylene bisacrylamide	20 g
Water to	1000 ml

Agarose (1 %)

Weighed 0.6 g of agarose powder and mixed with 60 ml of 1 X TAE buffer in a conical flask. Solution heated in a microwave oven until boiling and cooled slowly.

Ammonium persulphate (10%)

Ammonium persulphate	100 mg
Water to	1 ml

Denaturing Polyacrylamide Gel

0.5 X TBE Gelmix	60 ml
TEMED	0.125 ml
Ammonium persulphate (10 %)	0.125 ml

Mixed well without air bubbles

EDTA (0.5 *M*, pH 8.3)

Dissolved 18.61g of EDTA (disodium, dihydrate) in 80 ml of distilled water by bringing the pH to 8.3 with NaOH solution and volume made up to 100 ml. Stored at room temperature after filtration and autoclaving.

RBC lysis buffer

Ammonium chloride	150 mM	8.0235 g
Potassium chloride	10 mM	0.7455 g
EDTA	0.1 mM	0.0372 g

Dissolved the contents in distilled water and volume made up to 1000 ml. Stored at 4 °C after filtration and autoclaving.

Sodium acetate (3 M, pH 5.5)

Dissolved 40.824 g of sodium acetate in 70 ml of distilled water and pH adjusted to 5.5 with glacial acetic acid. Volume made up to 100 ml, autoclaved and stored at room temperature.

Sodium chloride (5 M)

Dissolved 29.22 g of sodium chloride in 80 ml distilled water and volume made up to 100 ml. Solution filtered and stored at room temperature.

Sodium chloride – EDTA (SE) buffer (pH, 8.0)

Sodium chloride	75 mM	4.383 g
EDTA	35 mM	9.306 g

Dissolved the contents in 900 ml distilled water and pH adjusted to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4 °C.

Sodium dodecyl sulphate (SDS) 20 %

SDS	20 g
Distilled water to make up to	100 ml

Ethidium Bromide (10 mg/ml)

Dissolved 100 mg ethidium bromide in10 ml of distilled water. Solution stored at 4 °C in a dark coloured bottle.

Formamide dye/ Stop buffer

Deionised formamide	98 %
Xylene cyanol	0.025 %
Bromophenol blue	0.025 %
0.5 M EDTA	10 m*M*

Gel loading buffer

Bromophenol Blue	0.25 %	50 mg
Xylene Cyanol	0.25 %	50 mg
Sucrose	40 %	8 g

Components stirred well in 20ml distilled water and stored at 4°C.

Phenol (Saturated, pH 7.8)

Commercially available crystalline phenol melted at 65 °C in a water bath. Hydroxyquinoline added to a final concentration of 0.1 percent. To the molten phenol, 0.5 M Tris HCl (pH 8.0) was added in equal volume and stirred for 30 min. on a magnetic stirrer. The contents were transferred into a separating funnel. Lower phenolic phase was collected and mixed with equal volume of 0.1 M Tris HCl (pH 8.0) and stirred again for 30 min. The phenolic phase was collected and extraction repeated with 0.1 M Tris HCl (pH 8.0) until the pH of phenolic phase was more than 7.8. Finally 0.1 volume of 0.01 M Tris HCl (pH 8.0) added and stored in a dark bottle at 4 °C.

RBC lysis buffer

Ammonium chloride	150 mM	8.0235 g
Potassium chloride	10 mM	0.7455 g
EDTA	0.1 mM	0.0372 g

Dissolved the contents in distilled water and volume made up to 1000 ml. Stored at 4 °C after filtration and autoclaving.

Sodium acetate (3 M, pH 5.5)

Dissolved 40.824 g of sodium acetate in 70 ml of distilled water and pH adjusted to 5.5 with glacial acetic acid. Volume made up to 100 ml, autoclaved and stored at room temperature.

Sodium chloride (5 M)

Dissolved 29.22 g of sodium chloride in 80 ml distilled water and volume made up to 100 ml. Solution filtered and stored at room temperature.

Sodium chloride – EDTA (SE) buffer (pH, 8.0)

Sodium chloride	75 mM	4.383 g
EDTA	35 mM	9.306 g

Dissolved the contents in 900 ml distilled water and pH adjusted to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4 °C.

Sodium dodecyl sulphate (SDS) 20 %

SDS	20 g
Distilled water to make up to	100 ml

Stirred, filtered and stored at room temperature.

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (P^H 8.0)	20 ml
Distilled water up to 1000 ml	
Autoclaved and stored at room temperature.	

Tris-Borate EDTA (TBE) buffer (pH 8.3) 10X

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

Dissolved in 700 ml of distilled water and pH adjusted to 8.3. Volume made up to 1000 ml, autoclaved and stored at room temperature.

TBE Gel mix (0.5X)

40 % Acrylamide	150 ml
10X TBE buffer	50 ml
Urea	450 g

Mixed well in 700 ml distilled water, volume made up to 1000ml and stored at 4 °C.

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140 mM	8.18 g
Potassium chloride	0.5 mM	0.0373 g
Tris base	0.25 mM	0.0303 g

Dissolved in 900 ml of distilled water and p^H adjusted to 7.4. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4 °C.

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 mM	1.2114 g
EDTA	0.1 mM	0.3722 g

Dissolved in 900 ml OF distilled water and adjusted the P^H to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4 °C.

Tris 1 M (pH 8.0)

Tris base	121.14 g

Added distilled water up to 1000 ml,. pH adjusted to 8.0, filtered and stored at room temperature.

ANNEXTURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICALS

Acrylamide (Molecular Biology grade)	-	SRL, Bombay
Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
Ammonium chloride	-	SRL, Bombay
Ammonium per sulphate	-	SRL, Bombay
N-N-Methylene Bis acrylamide	-	SRL, Bombay
Boric acid	-	SRL, Bombay
Chloroform	-	Merck
Crystalline phenol	-	Merck
Di-sodium hydrogen orthophosphate	-	SRL, Bombay
dNTPs.	-	Finn Enzymes
EDTA	-	SRL, Bombay
Ethanol	-	Merck
Ethidum bromide	-	BDH lab, England
6 X gel loading buffer	-	Bangalore Genei Pvt. Ltd.
Glacial acetic acid	-	BDH-E, Merck (India) Ltd.
Hydroxy quinolone	-	Qualigens Chemicals, Mumbai
Isoamyl alcohol	-	Merck
Methanol	-	SRL, Bombay
Potassium chloride	-	SRL, Bombay
Sodium acetate	-	SRL, Bombay
Sodium chloride	-	SRL, Bombay
Sodium dodecyl sulphate (SDS)	-	SRL, Bombay
TEMED	-	SRL, Bombay
Tris base	-	SRL, Bombay
Urea	-	SRL, Bombay

(B) PRIMERS

Imperial bio medics.

(C) MOLECULAR MARKERS

pBR322 DNA /*Hae*III digest	- Bangalore Genei Pvt. Ltd.
M13 sequencing ladder	- Amersham Pharmacia Biotech, USA.

(D) ENZYMES

Taq DNA polymerase	- Bangalore Genei Pvt. Ltd.
Proteinase-K	- Bangalore Genei Pvt. Ltd.
Polynucleotide kinase	- Bangalore Genei Pvt. Ltd.

(E) KITS

DNA-End-labelling kit	- Bangalore Genei Pvt. Ltd.
Sequenase version 2.0 DNA sequencing kit	- Amersham Pharmacia Biotech, USA.

(F) ISOTOPES

γ^{32} P-ATP	- BRIT, Bombay
α^{35} S-dATP	- BRIT (Jonaki), Hyderabad.

ANNEXURE – III

ABBREVIATIONS

RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
VNTR	Variable Number of Tandem Repeat
RAPD	Random Amplified Polymorphic DNA
DNA	Deoxyribo Nucleic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PIC	Polymorphic Information Content
EDTA	Ethylene Diamine Tetraacetic Acid
DTT	Dithiothretol
TEMED	N, N, N, N Tetramethylethylenediamine
APS	Ammonium Persulphate
PNK	Polynucleotide Kinase
FISH	Florescent *in situ* hybridization
SNP	Single Nucleotide Polymorphism
UPGMA	Unweighted Pair Group Method with Arithmetic means
FAO	Food and Agriculture Organization
ISAG	International Society of Animal Genetics
He	Heterozygosity
cM.	Centimorgan
µl	microlitres
µg	microgram
mg	milligram
mM.	millimolar
mCi	millicurie
Kb	Kilo base pair
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphate
dNTP.	Deoxy Nucleotide Triphosphate

ddATP.	Dideoxy Adenosine Triphosphate
ddCTP.	Dideoxy Cytosine Triphosphate.
ddGTP.	Dideoxy Guanosine Triphosphate
ddTTP.	Dideoxy Thymidine Triphosphate

MICROSATELLITE MARKER BASED CHARACTERIZATION OF INDIGENOUS PIGS OF KERALA

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ABSTRACT

The study was undertaken to assess the genetic diversity among four indigenous pig population of Kerala using microsatellite markers. The animals were selected from various part of Kerala, population I included the conserved Angamali pigs from university farm, Mannuthy, population II consisted of animals from Koothattukualm, population III were the animals from Ollur and animals from border districts of Kerala formed the population IV. Genetic analysis was carried out using five polymorphic microsatellite markers.

Blood samples were collected from 100 unrelated indigenous pigs from all four populations and DNA was isolated. The phenol-chloroform method of extraction yielded 224.35±9.86µg/5ml of blood. PCR conditions were standardized for all five selected markers namely, S0005, S0101, SW1026, SW2517 and S0008. The forward primer of each marker was endlabelled with γ32 P-ATP as source of radio signal. The M13 single strand DNA was sequenced and used as a size standard.

Autoradiography was employed to visualize the results. A total of eight alleles were detected in S0005 and S0101, five alleles in each of SW1026 and S0008, and six in SW2517. The heterozygosity varied from 0.7747 in SW2517 to as large as 0.8475 for S0005. The heterozygosity values for S0101, SW1026 and S0008 were 0.7774, 0.7672, and 0.7424 respectively. The PIC values ranges from 0.6974 for S0008 to 0.8291 for S0005. The PIC values for S0101, SW1026 and SW2517 were 0.7483, 0.7284 and 0.7381 respectively.

The allele frequencies were used to estimate the Nei's standard genetic distance among the populations. The distance measure ranged from 0.5704 to 0.7161, with the highest value noticed between population II and IV and the lowest between population I and III.

A dendrogram was constructed using the POPGENE version 3.2 program which grouped the population I and IV in one cluster and II and III populations in another cluster.